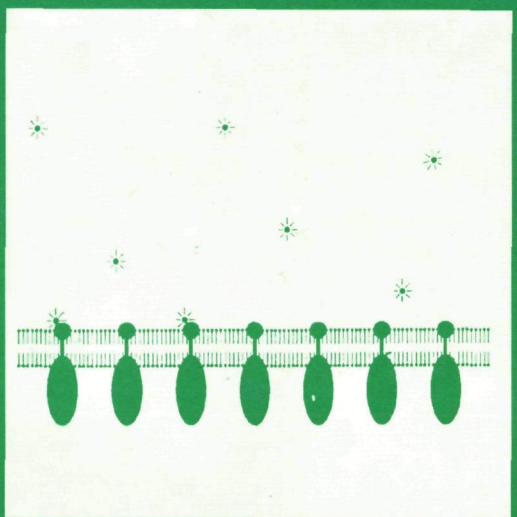
MEASUREMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR



C. Kienhuis

MEASUREMENT OF THE

EPIDERMAL GROWTH FACTOR RECEPTOR

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Een wetenschappelijke proeve op het gebied van
de Medische Wetenschappen,
in het bijzonder de geneeskunde

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The studies presented in this thesis were performed in the Department of Experimental and Chemical Endocrinology (head: prof.dr. Th.J. Benraad), University Hospital St. Radboud, Nijmegen, The Netherlands. This study represents the analytical part of a combined clinical and analytical study on epidermal growth factor receptor and its ligand(s) in human breast cancer. For the clinical part the reader is kindly referred to the doctoral thesis by P.G. Koenders: "Epidermal growth factor receptor and its ligand(s): associations with prognosis of patients with breast cancer", University of Nijmegen, The Netherlands, 1992.

CONTENTS

CHAPTER 1

INTRODUCTION AND SCOPE OF THE THESIS

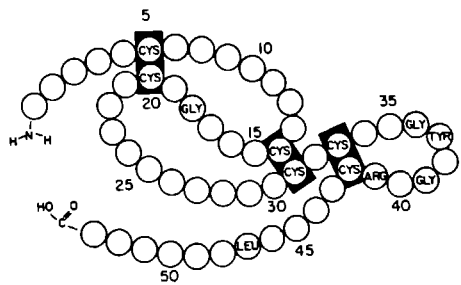
1.1 General introduction

Cellular proliferation and differentiation in the living organism are regulated at least in part by a complex network of interacting peptides, or polypeptide growth factors. The biology of these factors differs somewhat from classical hormones as neither their site(s) of synthesis nor site(s) of action is restricted to defined tissues. During the past decades, a considerable number of growth factors has been identified and isolated, such as epidermal growth factor (EGF), transforming growth factor alpha (TGFα) and -ß (TGF-ß), platelet-derived growth factor (PDGF), insulin and insulin-like growth factor I (IGF-I) and -II (IGF-II), and others (for reviews e.g. James and Bradshaw 1984, Carpenter 1987, Carpenter and Cohen 1990). As a result, it is now generally accepted that polypeptide growth factors represent a large family of regulatory agents, which interact on their target cells by high-affinity binding to specific cell surface receptors. The members of one group of growth factor receptors have in common that they contain intrinsic protein tyrosine kinase activity, by which the action of growth factors is mediated: upon binding of a growth factor to its receptor, a number of intracellular biochemical events is started that ultimately leads to the initiation of DNA replication and cell division (Hunter and Cooper 1985, Yarden and Ullrich 1988, Gill et al. 1988, Ullrich and Schlessinger 1990). Some growth factors can also have an inhibitory function, e.g. TGFß (Dickson and Lippman 1987, Dickson 1990).
Some growth factor receptors show a striking homology with particular oncogene products (Doolittle *et al.* 1983, Downward *et al.* 1984, Heldin and Westermark 1984, Hanks *et al.* 1988), which may interfere in receptor-mediated cellular responses. Constitutive or improper expression either of growth factors or of their receptors may result in loss of regulated growth and neoplastic transformation.
The results of an expanding number of investigations suggest that growth factors and their receptors are involved in the control of breast tumor growth (Barker and Vinson 1990, Dickson 1990). In this respect, a striking inverse relationship has been reported between the level of EGFR and the level of estrogen receptors (ER) in human primary breast tumors (Fitzpatrick *et al.* 1984, Pérez *et al.* 1984, Sainsbury *et al.* 1985, Skoog *et al.* 1986, Spitzer *et al.* 1987, Cappelletti *et al.* 1988, Delarue *et al.* 1988, Battaglia *et al.* 1988, Nicholson 1988, Pekonen *et al.* 1988, Foekens *et al.* 1989a, Fekete *et al.* 1989). High levels of EGFR in those tumors have been related to an early recurrence and a poor prognosis (Sainsbury *et al.* 1987, Rios *et al.* 1988, Grimaux *et al.* 1989, Foekens *et al.* 1989a, Nicholson *et al.* 1989). Increased expression of EGFR in neoplastic tissue might be an indicator of greater aggressiveness of the disease.

1.2 Epidermal growth factor

EGF was first identified and isolated from the mouse submaxillary gland in 1962 (Cohen 1962). The peptide was recognized by its ability to accelerate the eruption of teeth and the opening of eyelids of new born mice. The abundance of EGF in mouse salivary glands facilitated its purification and by 1972 the full amino acid sequence of mouse EGF had been determined (Savage *et al.* 1972). A few years later, a human urinary protein responsible for the inhibition of gastric acid secretion known as ß-urogastrone, was identified as the human EGF (Gregory 1975). The primary sites of EGF synthesis in mice are the salivary gland and the kidney, but the observation that

EGF can be found in milk and urine suggests that other sites may be important (Starkey and Orth 1977, Connolly and Rose 1988). In humans EGF has been found in most tissues (Kajikawa 1991).
Both mouse and human EGF are small polypeptides of 53 amino acid residues with a molecular mass of approximately 6000 daltons (Carpenter and Cohen 1979, 1990, Campbell *et al.* 1990). The three disulfide bonds in EGF between cysteine residues 6 and 20, 14 and 31, and 33 and 42, produce three disulfide loops in the secondary structure of the molecule (Savage *et al.* 1972, 1973) (Figure 1.1a).



a

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                    5         10        15        20        25        30
hEGF          N S Y P G C P S S Y D G Y C L N G G V C M Y I E A L D K Y A
mEGF          N S D S E C P L S H D G Y C L H D G V C M H I E S L D S Y T
hTGFα   V V S F H N D C P D S H T Q F C F H - G T C R F L V Q E D K P A
rTGFα   V V S H F N K C P D S H T Q Y C F H - G T C R F L V Q E E K P A
VGF     D I P A I R L C G P E G D G Y C L H - G D C I H A R D I D G M Y
AR      . . . . . N P C N A E F Q N F C I H - G E C K Y I E H L E A V T
                    *                 *         *   *

                   35        40        45        50
hEGF          C N C V V G Y I G E R C Q Y R D L K W W E L R
mEGF          C N C V I G Y S G D R C Q Y R D L K W W E L R
hTGFα         C V C H S G Y V G A R C E H A D L L A
rTGFα         C V C H S G Y V G V R C E H A D L L A
VGF           C R C S H G Y T G I R C Q H V V L V D Y Q
AR            C K C Q Q E Y F G E R C G E K
              *   *       *   *   * *       *
```

b

Fig. 1.1 *a, The mouse EGF molecule; b, Alignment of the sequences of some members of the EGF-family (h= human; m= mouse; r= rat); VGF= Vaccinia growth factor; AR= Amphiregulin. Amino acids are represented by standard one-letter symbols (A=Ala; C=Cys; D=Asp; E=Glu; F=Phe; G=Gly; H=His; I=Ile; K=Lys; L=Leu; M=Met; N=Asn; P=Pro; Q=Gln; R=Arg; S=Ser; T=Thr; V=Val; W=Trp; Y=Tyr). Conserved amino acid residues are marked with an asterisk. Hyphen indicate gap introduced to maximize homology.*

These disulfide bonds in EGF are the most conserved structural characteristic among EGF-like peptides (Carpenter and Wahl 1990) (Figure 1.1b) and are required for biological activity (Taylor *et al.* 1972). In addition, further studies have demonstrated a nearly absolute requirement for Arg-41 (Engler *et al.* 1990), and the importance of the hydrophobic Leu-47 (Engler *et al.* 1988) in receptor binding. Furthermore, other amino acid residues, such as Val-19, Met-21, Ile-23, Leu-26, and Tyr-29, have been proposed to play an important role in ligand-receptor interactions (Engler *et al.* 1990, 1991, Campion *et al.* 1990).
From analysis of the cDNA sequence, the precursor (preproEGF) for mouse EGF is predicted to be a 1217 amino acid transmembrane protein with an extracellular domain that contains at least seven EGF-like sequences (Gray *et al.* 1983, Scott *et al.* 1983, Doolittle *et al.* 1984). The human EGF precursor is a 1207 amino acid molecule with 66% homology with the mouse protein (Bell *et al.* 1986). The EGF precursor may be proteolytically cleaved to biologically active lower molecular weight fragments (Breyer and Cohen 1990).
EGF has been demonstrated to elicit significant biological responses in intact animals, organ cultures, and cell culture systems (Carpenter and Cohen 1990, Burgess 1989). The in vivo functions of EGF are assumed to be stimulation of fetal growth and development, regulation of growth and differentiation of continuously regenerating tissues, and stimulation of wound healing processes (Fisher and Lakshmanan 1990).

1.3 Epidermal growth factor-like peptides

Based on their structural homologies, polypeptide growth factors can be grouped into different families. Each family has probably arisen from common ancestor genes by duplications and evolutionary divergence (Yarden and Ullrich 1988). One growth factor family is constituted by the EGF-family (Carpenter and Wahl 1990) that includes EGF, TGFα (Massague 1983, Derynck *et al.* 1984), vaccinia growth factor (VGF) (Stroobant *et al.* 1985), amphiregulin (Shoyab *et al.* 1989, Plowman *et al.* 1990), cripto (Ciccodicola *et al.* 1989), and a heparin-binding factor from macrophages (Higashiyama *et al.* 1991). The EGF-like peptide growth factors are all encoded by separate genes, but each one is structurally analogous to the EGF sequence, compete with ^{125}I-EGF in radioreceptor assays, and produce EGF-like biological responses in sensitive cells (Donaldson *et al.* 1989, Burgess 1989, Carpenter and Wahl 1990, Campbell *et al.* 1990).
TGFα, originally isolated from retrovirus-transformed cells (Delarco and Todaro 1978) is an important member of the EGF-family, since it has been proposed to play an important role in carcinogenesis (Dickson and Lippman 1987). A large variety of tumor cells indeed do synthesize TGFα (Derynck *et al.* 1987, Dickson 1990). Comparative studies of the effects of both EGF and TGFα have established that both growth factors evoke similar actions, but that TGFα is more potent than EGF in a variety of biological systems (Stern *et al.* 1985, Ibbotson *et al.* 1986, Gan *et al.* 1987). This observation is not explained by differences in affinities of the ligands for the receptor, because the affinity constants of both factors are similar (Todaro *et al.* 1980, Massague *et al.* 1983, Winkler *et al.* 1986, Lax *et al.* 1988a). Differences in binding (Lax *et al.* 1988a, Winkler *et al.* 1989) or differences in processing of ligand-receptor complexes (Ebner and Derynck 1991) may explain why TGFα exerts quantitatively higher activities than EGF.

1.4 The epidermal growth factor receptor

The first step in the action of EGF is specific binding in a 1:1 stoichiometry (Weber *et al.* 1984) to a surface glycoprotein known as the EGF receptor, a single chain membrane-bound glycoprotein composed of 1186 amino acid residues (Mr = 170,000), and a substantial amount of N-linked oligosaccharide (Carpenter 1987, Schlessinger 1988a,b, Donaldson *et al.* 1989, Velu 1990). The receptor is, like other growth factor receptors, composed of an extracellular ligand-binding domain connected by a single hydrophobic region to the cytoplasmic protein tyrosine kinase domain (Fig. 1.2) (Carpenter 1987, Schlessinger 1988a,b, Hsuan *et al.* 1989a,

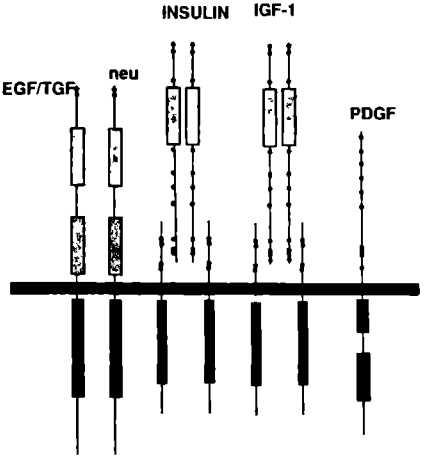

Fig. 1.2. *Schematic representation of the EGF/TGF α receptor and other ligand activated tyrosine protein kinases. The hatched boxed and black dots indicate cysteine rich homologous regions The black boxes represent the tyrosine kinase region IGF-1, insulin-like growth factor-1; PDGF, platelet derived growth factor. (adapted from Waterfield, 1989).*

Waterfield 1989). The cytoplasmic domain of the EGF receptor bears a striking similarity to the transforming v-erbB protein of the Avian Erythroblastosis Virus (Downward *et al.* 1984). This virus transforms cells by introducing a truncated EGF receptor into the cells. This truncated EGFR lacks most of the external EGF-binding domain and provides a mitogenic signal in the absence of exogenous EGF.
The extracellular domain of the EGF receptor (621 amino acids), which contains 10-11 N-linked oligosaccharide chains (Cummings *et al.* 1985), can be subdivided into four subdomains (Lax *et al.* 1988b, 1989, 1990). According to this assignment, domain I is the amino-terminal domain, domains II and IV are two cysteine-rich domains, and domain III is located between these latter cysteine-rich domains and possesses significant homology with domain I (Lax *et al.* 1988b). Domain III has been shown to be a major ligand-binding domain (Wu *et al.* 1989, 1990, Lax *et al.* 1988b). The added

contributions of both domain I and III to the binding energy generate the high-affinity binding site typical of human EGFR (Lax *et al.* 1991a). Electron microscopic studies suggested the ligand binding domain to be located in the cleft between domain I and III, while the two cysteine-rich domains are in contact with each other and close to the plasma membrane (Lax *et al.* 1991b).

The ligand-binding domain of the EGF receptor is separated from the cytoplasmic domain by a single transmembrane segment of 23 uncharged, hydrophobic amino acid residues (622-644) (Ullrich *et al.* 1984), which is anchored intracellularly by a highly basic, stoptransfer sequence (645-657). The functional role of this domain in transmission of the mitogenic signal has not been established. Mutations within the transmembrane domain do not affect receptor function (Kashles *et al.* 1988, Carpenter *et al.* 1991). In contrast, mutations within the equivalent domain of the EGFR-related rat *neu* or c-*erbB2* putative receptors stimulate the kinase activity and can lead to cellular transformation (Bargmann *et al.* 1986, Bargmann and Weinberg 1988). The observation that an alteration of the lipid environment in cell lysates can modify both ligand affinity and kinase activity provides further evidence for a possible functional role of the transmembrane domain (Downward *et al.* 1985).

The cytoplasmic domain of the receptor (542 amino acids) contains a tyrosine kinase domain, which is the most highly conserved portion of all receptor tyrosine kinase molecules (Hanks *et al.* 1988, Ullrich and Schlessinger 1990, Yarden and Ullrich 1988). Lysine residue 721 within this region binds ATP, generating the phosphate for the tyrosine phosphorylation reaction. Replacement of this lysine residue in EGFR abolishes the tyrosine kinase activity both in vitro and in vivo (Honegger *et al.* 1987a, Chen *et al.* 1987). Various mutant EGFRs devoid of kinase activity have normal ligand binding characteristics, but are unable to stimulate various responses of EGF (Livneh *et al.* 1986, 1987, Chen *et al.* 1989, Honegger *et al.* 1987a,b, Moolenaar *et al.* 1988). This suggests that tyrosine phosphorylation of cellular substrates by the receptor tyrosine kinase is essential for transduction of the mitogenic signal. This suggestion is confirmed by the observation that mutation of the EGFR molecule which removes three C-terminal conserved tyrosine residues results in a receptor that is almost inactive biologically (Helin *et al.* 1991, Helin and Beguinot 1991, Magni *et al.* 1991). The tyrosine kinase activity of the EGF receptor has also been found to be necessary for normal receptor targeting to lysosomes (Honegger *et al.* 1987a, Glenney *et al.* 1988, Chen *et al.* 1989, Felder *et al.* 1990). Kinase-defective receptors are internalized, but are recycled to the cell surface for reutilization (Honegger *et al.* 1990). It is not known whether this means that lysosomal targeting requires phosphorylation of particular substrates.

In cells expressing EGFR the major substrate of the intrinsic tyrosine kinase is the receptor itself: the main phosphorylation sites are four tyrosine residues located in the C-terminal region of the intracellular domain, which become phosphorylated after ligand binding (Downward *et al.* 1984, Yarden and Schlessinger 1987a, Yarden and Ullrich 1988, Hsuan *et al.* 1989b, Margolis *et al.* 1989a, Lai *et al.* 1989b). Point mutation analyses of these autophosphorylation sites have shown only minor differences in receptor activities of mutant versus wild type receptors. Recent studies suggest that autophosphorylation sites have a regulatory function for the kinase activity in that they compete with exogenous substrates for the substrate binding site of the tyrosine kinase domain (Honegger *et al.* 1988a,b, Bertics *et al.* 1988, Margolis *et al.* 1990). Autophosphorylation seems to be mediated by intermolecular cross-

phosphorylation within a oligomeric receptor complex (Schlessinger 1988b).
Another regulatory site is Thr-654, which is phosphorylated by protein kinase C-mediated phosphorylation (Hunter *et al.* 1984, Davis and Czech 1985). Mitogens that activate protein kinase C reduce EGF binding affinity and inhibit EGF receptor tyrosine kinase activity (Shoyab *et al.* 1979, Cochet *et al.* 1984). It has been proposed that the phosphorylation of Thr-654 allosterically regulates ligand binding affinity and tyrosine kinase activity of the EGF receptor (Hunter and Cooper 1985). Phosphorylation of EGFR at Thr-654 leads to downregulation of the tyrosine kinase activity and to rapid physiological attenuation of the mitogenic response to EGF (Davis 1988, Livneh *et al.* 1988, Lund *et al.* 1990, Welsh *et al.* 1991), but does not lead to downregulation of high-affinity binding of the receptor (Livneh *et al.* 1988). The latter effect might be induced by phosphorylation of other residues on the receptor.

1.5 Signal transduction

The binding of EGF to its receptor is the initial event in the stimulation of cell growth induced by this growth factor. In the absence of EGF, EGF receptors are randomly distributed on the cell surface (Schlessinger *et al.* 1978, Haigler *et al.* 1978, Haigler and Cohen 1979). After ligand binding, the receptors rapidly aggregate in clathrin-coated pits and are internalized via endosomes (Carpenter and Cohen 1979, Lai *et al.* 1989a). The ligand then dissociates from its receptor in the acidic environment of the endosomes. Following fusion with lysosomes, EGF and EGFR are rapidly degraded (Carpenter and Cohen 1979).
Binding of EGF to the extracellular receptor domain is proposed to result in receptor oligomerization (Schlessinger 1988a,b, Cochet *et al.* 1988), followed by intermolecular activation of the intrinsic protein tyrosine kinase domain (Schlessinger *et al.* 1983, Schlessinger 1986, Yarden and Schlessinger 1987a,b, Lammers *et al.* 1990). The ligand-induced dimerization seems to induce higher ligand binding affinity. It has been hypothesized that oligomeric receptors show high affinity binding, while single receptors have low affinity (Böni-Schnetzler *et al.* 1987, Schlessinger 1988a), and therefore, the binding of EGF will stabilize the oligomeric state, which leads to the activation of the kinase domain.
Several proteins are phosphorylated by the EGF receptor, e.g. phospholipase C-τ (PLC-τ) (Margolis 1989b, Wahl 1989). After ligand binding, EGFR becomes associated with PLC-τ (Kim *et al.* 1990, Margolis *et al.* 1990). The phosphorylation of PLC-τ apparently activates the enzyme and triggers hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), which generates two intracellular second messengers: diacylglycerol (DAG) and inositol 1,4,5,-triphosphate (IP_3) (Hepler *et al.* 1987, Nishibe *et al.* 1990, Payrastre *et al.* 1991). DAG stimulates protein kinase C (PKC) which in turn phosphorylates the EGF receptor at Thr654 and at other residues and activates the Na^+-H^+ exchange (Nishizuka 1988). IP_3 binds to its receptor (IP_3-R) at the surface of an intracellular Ca^{2+} storage organelle and induces release of stored Ca2+ to the cytoplasm and Ca^{2+} influx, increasing $[Ca^{2+}]_i$ (Chen *et al.* 1987, Pandiella *et al.* 1989, Hepler *et al.* 1987). Ultimately, the EGF initiated signal leads to increased nuclear activity, which includes induction of synthesis of specific mRNA (c-fos, c-myc, and c-jun proto-oncogenes) and DNA (Müller *et al.* 1984, Bravo *et al.* 1985, Quantin *et al.* 1988).

1.6 Epidermal growth factor receptors in breast cancer

Estrogens play an important role in the development and progression of human breast cancer. In the normal breast it acts as a mitogen and induces the progesterone receptor (PgR) which is responsible for the development and differentiation of the breast (Horwitz *et al.* 1985). It has generally been accepted that ER levels in breast tumors define the hormone dependency of the tumor and predict those patients who have a good prognosis and who are likely to benefit from endocrine therapy (King 1990, Barker and Vinson 1990). However, an enigma of the human breast cancer work is that many ER-positive tumors do not respond to endocrine therapy, i.e. there remains a large percentage (50%) of ER positive tumors who fail to respond (King 1990). A great deal of evidence has arisen to suggest that the mitogenic effects of oestrogens are mediated in some part via peptide growth factors and their receptors (Lippman and Dickson 1989).

The majority of information on the mechanism of action of growth factors and their receptors has been obtained in studies with human breast cancer cells in vitro. The proliferative response to estrogen is accompanied by increased expression of a number of growth factor genes (EGF, TGFα and -β, IGF-I and -II, PDGF) that are thought to control proliferation via autocrine and paracrine mechanisms (Mori *et al.* 1986, Dickson and Lippman 1987, Bates *et al.* 1988). Information regarding possible interactions between ER and EGFR through the action of their respective ligands has so far been contradictory. Studies using MCF-7 human breast cancer cells indicate that estrogen can cause an increase in EGFR levels with a concomitant decrease in ER, and that the anti-estrogen hydroxytamoxifen causes a decrease in EGFR (Berthois *et al.* 1989). Treatment of these cells with EGF was shown to increase ER and PgR, with a corresponding decrease in EGFR levels, inferring a parallel inverse relationship to that found in breast tumor biopsies. However, since binding of ligand to EGFR causes receptor downregulation and TGFα production is stimulated by estrogen and inhibited by anti-estrogen treatment (Lippman and Dickson 1989), it is not clear how far such a parallel extends (Barker and Vinson 1990).

Apart from their effects on cell proliferation in vitro, various effects of growth factors and their receptors have been observed to be mediated by progestins. Incubation of human breast cancer cells with progestins increased in a time and dose-dependent manner the amount of EGFR, both at the protein and the mRNA level (Murphy *et al.* 1985, Murphy *et al.* 1988a, Ewing *et al.* 1989), and of its ligands EGF and TGFα (Murphy *et al.* 1988b, Murphy and Dotzlaw 1989). Under these in vitro conditions EGF plays a dominant role (Sarup *et al.* 1988), capable of reversing any growth inhibitory effects of progestins. In vivo, EGF as well as TGFα are probably produced locally in many tissues as local growth factors rather than as systemic hormones (Dickson and Lippman 1987). There is evidence that EGF plays a role in carcinogenesis and that the EGF-stimulated growth regulatory system is also involved in proliferation of malignant cells (Stoscheck and King 1986), but the majority of evidence indicates that TGFα, via the EGF receptor, and IGF-I promote cell proliferation, while TGF-β is thought to be a growth inhibitor (Dickson and Lippman 1987).

In the search for prognostic factors in human breast cancer in addition to ER, EGF receptors have been demonstrated on breast cancer cells, especially on ER-negative tumor cells, and in human primary breast tumors and their metastases (Davidson *et al.* 1987). An increase in EGFR has been proposed as a mechanism by which

unopposed estrogen might play a role in the etiology of breast cancer and as an additional prognostic factor in human breast cancer. In human primary breast tumors, based on 40 separate studies comprising more than 5,000 patients, the mean percentage of EGFR-positivity was ±45% (Klijn *et al.* 1992). Gene amplification and mRNA overexpression of EGFR or the closely related erbB2 oncogene have also been found in mammary carcinomas (Klijn *et al.* 1992). Moreover, EGFR has been identified in a wide variety of tissues (O'Keefe *et al.* 1974), including all histological types of breast tissue (Barker *et al.* 1989, Möller *et al.* 1989). At present there is no agreement on the clinical relationships and prognostic value of EGFR in human breast cancer. The levels of EGFR and its incidence as described in the literature vary widely (P.G. Koenders, doctoral thesis University of Nijmegen, 1992). Nearly all reported studies indicate a negative relationship between EGFR and ER status, and PgR status (Fitzpatrick *et al.* 1984, Pérez *et al.* 1984, Sainsbury *et al.* 1985, Skoog *et al.* 1986, Spitzer *et al.* 1987, Cappelletti *et al.* 1988, Delarue *et al.* 1988, Battaglia *et al.* 1988, Pekonen *et al.* 1988, Foekens *et al.* 1989a, Fekete *et al.* 1989). Interestingly, it has been reported that the tumor's EGFR status could predict an early recurrence and death, and EGFR positivity of the tumor was associated with a lack of response to endocrine therapy (Sainsbury *et al.* 1987), but the results correlating EGFR status and relapse-free survival are very controversial and the prognostic value of EGFR for length of (relapse-free) survival in breast cancer is currently a matter of debate.

1.7 Scope of the thesis

As was stated above, the levels of EGFR and its incidence in human primary breast tumors in various studies vary widely. This may have contributed essentially to the highly controversial reports regarding the prognostic value of EGFR. Methodological differences provide plausible explanations for this disturbing variation. Assay conditions differ widely with respect to tissue processing, the procedure for the radioiodination of EGF, the incubation conditions, the ligand concentrations used in multiple point and single saturation dose analysis, and the method of separation of receptor-bound from unbound ligand. For application in clinical studies, strict assay conditions should be defined and the availability of a reproducible and reliable assay is imperative. The European Organization for Research and Treatment of Cancer (EORTC) Receptor Study Group urged our department to develop an EGFR assay which stands under rigorous conditions. This thesis concerns the development of such an assay and its evaluation in all its aspects. These aspects involve the establishment of strict assay conditions, such as time and temperature of the assay, ligand concentrations, and the preparation of the radioactive ligand. For separation of receptor-bound from free ligand, hydroxylapatite (HAP) was used. The use of HAP circumvents the employment of high-speed centrifugation for the separation step (Benraad and Foekens 1990).
EGF receptor function depends on its ligands EGF and TGFα. Therefore, besides measurement of EGFR, it is of major interest to assess the concentration of its ligand(s). This thesis describes a radioreceptor assay (RRA) to measure the levels of EGFR ligand(s) in tumor cytosols. The RRA procedure was also based on the HAP bound-free separation. Furthermore, employing a specific rabbit anti-mouse EGF antibody, a radioimmunoassay to measure mEGF could be developed.

The analytical methods described were applied to investigate the role of the EGF/ TGFα - EGFR pathway in breast cancer using two different approaches. Firstly, human primary breast tumors were analyzed for EGFR and EGFR ligand(s) in addition to steroid hormone receptors, which is described in the doctoral thesis by P.G. Koenders ("Epidermal growth factor receptor and its ligand(s): associations with prognosis of patients with breast cancer", University of Nijmegen, 1992). The data obtained were examined in relation to tumor and patient characteristics and in relation to prognosis of patients with breast cancer. Secondly, in the present thesis the analytical methods described were used to investigate the relationship of the levels of EGFR and EGFR ligand(s) with the levels of steroid hormone receptors in a mouse mammary tumor model. In this model progression of tumors to hormone independence is observed in serial transplants of tumors that are induced in ovariectomized female GR strain mice by continuous administration of estrone and progesterone (Sluyser and Van Nie 1974, Sluyser *et al.* 1976). The induced tumors are initially hormone dependent, but during transplantation they become hormone responsive and finally hormone independent.

References are listed on pages 99-110.

CHAPTER 2

DEVELOPMENT OF A RADIOLIGAND BINDING ASSAY FOR THE MEASUREMENT OF THE EPIDERMAL GROWTH FACTOR RECEPTOR

2.1 Introduction

Many studies have been reported analyzing EGFR levels in primary human breast tumors and/or whether this level can serve as a prognostic factor in human breast cancer (for reviews: Klijn *et al.* 1992, P.G. Koenders, doctoral thesis, University of Nijmegen, 1992). It is highly disturbing however, that the percentages of EGFR positivity as reported in these vast number of publications vary from 15% to 91% This large variation can partly be ascribed to differences in methodology (Dittadi *et al.* 1990b). Assay conditions differ widely with respect to the preparation of the membrane fraction, the radioiodination procedure of EGF, the ligand concentrations used in multiple point and single point saturation dose analysis, the incubation conditions such as time and temperature, and the method of separation of receptor-bound from unbound ligand (Table 2.1). To restrict the variation in EGFR assay results between laboratories, it was decided to establish an efficient analytical method which under rigid assay conditions gives reproducible results with high comparability between laboratories. As many routine laboratories do not have the facilities to employ a method dependent on high speed centrifugation to separate receptor-bound from free ligand on a large scale, an assay was introduced using hydroxylapatite (HAP) to achieve this separation in a more convenient way. An outline of this HAP procedure was reported earlier (Benraad and Foekens 1990).

HAP, a form of calcium phosphate, is widely used in preparative biochemistry for the fractionation and purification of monoclonal antibodies (Stanker *et al.* 1985, Smith *et al.* 1984) and other proteins (Engel *et al.* 1980) and nucleic acids (Kantler and Schwartz 1979, Johnson and Ilan 1983). Binding of proteins to HAP occurs by attraction between the positive charges of the protein and HAP, and by complexing of protein carboxyls with calcium loci on the mineral (Gorbunoff 1984a,b, Gorbunoff and Timasheff 1984). Low molecular weight substances, such as amino acids, are poorly or not at all bound to HAP.

HAP has already been used in steroid hormone receptor assays (Erdos *et al.* 1970, Smith *et al.* 1979, Garola and McGuire 1978). In the case of the EGFR assay, HAP adsorps the ligand-EGFR complex which is present in the membrane fraction leaving the nonbound ligand in solution. One obtains this membrane fraction when tumor tissue is processed for routine steroid hormone receptor determinations in the cytosolic fraction (Benraad and Foekens, 1990).

In this chapter, a rigorous analyse of the assay conditions of the radioligand binding assay of EGFR using HAP is described. These include for example time and temperature required for reaching equilibrium in the ligand-receptor reaction, time and temperature to be used during the HAP separation step, measures adopted to reduce nonspecific binding and thereby to increase assay sensitivity, and choice of the range of ligand concentrations used in the multiple point tracer analysis.

Table 2.1. *Variance in EGFR ligand binding assay methodology in breast cancer*

Author	Membrane fraction	Iodination method[1]	[EGF] (nM)[2]	Incubation conditions	Separation bound-free[3]
Fitzpatrick (1984)	105,000 x g -2,000 x g	Chl T	1.0	1 h 25°C	Gelman GA-8
Pérez (1984)	100,000 x g -12,000 x g	Chl T	0.1-10 comp.	1 h 20°C	3,000 x g
Skoog (1986)	100,000 x g -12,000 x g	Chl T	10^5 cpm comp.	1 h 20°C	3,000 x g
Sainsbury (1985)	100,000 x g -1,000 x g	Iodogen	0.6 comp.	2 h 26°C	14,000 x g
Weisman (1987)	100,000 x g -7,000 x g	Chl T	0.2	16 h 25°C	2,300 x g
Wyss (1987)	100,000 x g -1,000 x g	Chl T*	3.6	2 h 25°C	Millipore HVLP
Battaglia (1988)	105,000 x g -7,000 x g	unknown	2.6 0.4-2.6	16 h 20°C	2,000 x g
Cappelletti (1988)	40,000 x g -800 x g	Chl T	5.0	1 h 25°C	Whatman GF/B
Delarue (1988)	100,000 x g -800 x g	Iodogen	0.3-1.2	2 h 25°C	15,000 x g
Nicholson (1988)	100,000 x g -800 x g	Iodogen	1.0 0.15-10	1 h 26°C	14,000 x g
Pekonen (1988)	30,000 x g -800 x g	Chl T	0.1	1 h 37°C	30,000 x g
Rios (1988)	100,000 x g -12,000 x g	unknown	0.6 comp.	1 h 20°C	3,000 x g
Barker (1989)	100,000 x g -800 x g	unknown	1.0	2 h 26°C	10,000 x g
Bauknecht (1989)	5,000 x g -100 x g	Chl T	0.4	45 min 37°C	Cellulose-acetaat
Fekete (1989)	105,000 x g -500 x g	Chl T	0.4 comp.	3 h 21°C	3,000 x g
Foekens (1989a)	50,000 x g -8,000 x g	Chl T	0.8 0.1-0.8	16 h 20°C	30,000 x g
Grimaux (1989)	105,000 x g -7,000 x g	Chl T	3.0	2 h 25°C	3,000 x g
Harris (1989)	100,000 x g -800 x g	Iodogen	1.0	1 h 26°C	14,000 x g
Llorens (1989)	100,000 x g -800 x g	Iodogen	0.03-0.5	2 h 25°C	5,000 x g
Lyonnais (1989)	100,000 x g -800 x g	LP	0.2-1.6	45 min 25°C	10,000 x g
Bolla (1990)	25,000 x g -1,500 x g	Chl T	1.0	1 h 25°C	Whatman GF/C
Bolufer (1990)	25,000 x g -1,000 x g	Chl T*	0.5 0.015-0.15	2 h 20°C	20,000 x g
Costa (1990)	100,000 x g -1,000 x g	unknown	3.6	2 h 4°C	Millipore
Dittadi (1990a)	100,000 x g -800 x g	LP	0.03-3.0	20 h 26°C	mAbR1/4,000 x g

Formento (1990)	105,000 x g -800 x g	LP	1.0	1.5 h 22°C	11,000 x g
Spyratos (1990)	100,000 x g -800 x g	Iodogen	0.3-1.2	2 h 25°C	15,000 x g
Toi (1990)	105,000 x g -2,000 x g	Chl T	0.05-5.0	1 h 25°C	Gelman GA-8
Falette (1992)	48,000 x g -800 x g	Iodogen	0.5 10	1 h 20°C	3,000 x g

[1] *Chl T: Chloramine T; Chl T*: probably Chloramine T; LP: Lactoperoxidase.*
[2] *In those cases one concentration is mentioned here, a single saturation dose is used; a range of concentrations denotes a multiple point titration method; comp: competition between one concentration of ^{125}I-EGF and varying concentrations of unlabeled EGF.*
[3] *g-force (centrifugation) or type of filter (ultrafiltration) used.*

2.2 Materials and methods

2.2.1 Materials

Receptor grade mouse EGF was obtained from Bioproducts for Science, Inc. (Indianapolis, IN, U.S.A.). Carrier-free $Na^{125}I$ was obtained from Amersham International plc. (Amersham, Buckinghamshire, U.K.). Iodine monochloride (ICl) was obtained from Merck (Darmstadt, Germany). Enzymobeads and hydroxylapatite (HAP; DNA grade Biogel HTP) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). β-D-Glucose was supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bacitracin was obtained from Serva (Heidelberg, Germany). Bovine serum albumin (BSA) was purchased from Behringwerke AG (Marburg/Lahn, Germany). Protag-125 and disposable 3-mL C_{18} solid-phase extraction (SPE) columns (200 mg of C_{18}) were obtained from J.T. Baker, Inc. (Phillipsburg, NJ, USA).

2.2.2 Preparation of membranes

2.2.2.1 Human placental membranes (HPM)

Placental tissue was dissected free of total membranes and fleeces and homogenized in 1 ml of ice-cold Tris buffer (20 mM Tris-HCl, 250 mM sucrose, 2 mM $MgCl_2$, 2 mM KCl, pH 7.6) per gram of tissue for 10 seconds, three times, in a Waring Blendor (high setting). After centrifugation for 20 min at 800 x g, the pellet was rehomogenized in an equal volume of Tris buffer with the Waring Blendor and centrifuged for 20 min at 800 x g. The pellet obtained after this centrifugation step was discarded. The supernatant fractions from both centrifugation steps were pooled and centrifuged for 30 min at 10,000 x g, and the resulting pellet was also discarded. The membrane fraction was obtained by centrifugation of the supernate for 40 min at 45,000 x g. The pellet was resuspended in 1 ml of PBB-buffer (20 mM phosphate, 150 mM NaCl, 50 μM bacitracin, pH 7.4) per gram of placenta tissue (initial weight). All procedures were performed at 0-4 °C. The membrane preparation was stored at -80 °C in 1 ml-aliquots.

2.2.2.2 Human breast tumor membranes

Human breast tumor membranes were obtained during routine processing of tumor biopsy samples for steroid hormone receptor assays (EORTC 1980). Breast tumor biopsies were pulverized in the frozen state in a microdismembrator (Braun, Melsungen, Germany) and homogenized in 5 ml of steroid hormone receptor assay buffer (10 mM K_2HPO_4/KH_2PO_4, 1.5 mM dipotassium EDTA, 3 mM NaN_3, 10 mM monothioglycerol, 10%, v/v, glycerol, pH 7.4) per gram of tumor powder. The crude homogenate was centrifuged for 10 min at 800 x g (4 °C). The supernate was centrifuged for 1 h at 105,000 x g (4 °C). The pellet was rinsed with 1 ml of ice-cold PPB-buffer and stored at -80 °C under an aliquot of PBB-buffer. The membrane pellet was resuspended in 1100 µl of PBB-buffer by sonication (3x5 sec, Soniprep 150 MSE, Scientific Instruments, Crawley, U.K.). Prior to the binding studies a 100 µl aliquot of the membrane fraction was taken out for protein determination (Bradford 1976), after which a solution of 10% of BSA in PBB-buffer was added to the membrane preparation to a final concentration of 0.1% of BSA.

2.2.3 Radioiodination of EGF

EGF was radioiodinated by using either Protag-125, iodine monochloride (ICl), or Enzymobeads to a specific radioactivity of about 500 Ci/mmol (100 µCi/µg). The details of these iodination procedures are described in chapter 4.

2.2.4 Preparation of HAP suspension

The HAP suspension was prepared according to the manufacturer's instructions. Approximately 40 ml of PBB-buffer without BSA was added to about 5 g of HAP powder. Course HAP particles were eliminated by repeatedly settling the HAP suspension for 15 sec at gravity and decanting the suspension into another tube, leaving the course particles in the first one. The remaining HAP slurry was washed several times with PBB-buffer until the pH of the supernate was 7.4. Finally, PBB-buffer was added to the HAP pellet in a HAP:buffer ratio of 2:1 (v/v).

2.3 Results

2.3.1 Nonspecific adsorption of ^{125}I-EGF to HAP (Blank HAP binding)

The reliability of the HAP assay strongly depends on the efficiency of the separation of bound and free ^{125}I-EGF. Any amount of ^{125}I-EGF remaining adsorbed to HAP contributes to the nonspecific binding and therefore should be avoided as much as possible, thereby increasing assay sensitivity. Therefore, the capability of various substances in reducing the blank HAP binding was investigated. Table 2.1 shows that a large amount (almost 65%) of the ^{125}I-EGF was adsorbed to HAP when the buffer only contains phosphate and sodium chloride (phosphate buffered saline, PBS), even after washing of the HAP twice with PBS. In the presence of 0.1% of BSA, the blank

Table 2.1 Effect of BSA and bacitracin on blank HAP binding of ^{125}I-EGF

Addition to assay buffer	Addition to HAP	Blank HAP binding (%)
-BSA/-bac	-bac	64 9 ± 1 9
+BSA/-bac	-bac	5.1 ± 0 7
+BSA/+bac	+bac	0 6 ± 0 02

About 1 5 x 10^5 cpm of ^{125}I-EGF in 140 µl of PBS or PBS containing BSA (0 1%) and/or bacitracin (50 µM) was incubated with 100 µl of HAP suspension for 30 min at 20 °C The HAP suspension was prepared in PBS with or without 50 µM bacitracin Blank HAP binding is represented as the % of the total radioactivity added (mean ± S D , n=5) The ^{125}I-EGF preparation was preincubated with 200 µl of HAP suspension for 30 min at 20 °C with HAP After centrifugation for 2 min at 800 x g, the resulting tracer preparation was recovered in the supernate This HAP purification reduced the blank HAP binding of the ^{125}I-EGF (See also chapter 4) BSA= bovine serum albumin, bac= bacitracin

HAP binding decreased to about 5% of total ^{125}I-EGF added When in addition bacitracin (50 µM) was present in the buffer, the blank HAP binding reduced further to about 0 6% under the conditions of this experiment, which included two HAP washing steps with buffer Additional washing steps did not further reduce the blank HAP binding Increasing the concentration of bacitracin to 75 and 100 µM did not further decrease the blank HAP binding

Insulin (50 µM) was equally effective as bacitracin in reducing the blank HAP binding, but other proteins tested, i e vancomycine, colimycine, streptomycine, cystatine, and vasopressine, were less effective in reducing blank HAP binding (data not shown)

As expected, the blank HAP binding increased with increasing volumes of HAP suspension added The amount of nonspecific binding detected was directly proportional to the volume of HAP suspension used In the EGFR assay ultimately developed, 100 µl of HAP suspension was employed, adding it to the 140 µl incubation mixture 0 1% of BSA and 50 µM bacitracin were included in the assay buffer, and 50 µM bacitracin was included in the HAP suspension buffer

2 3 2 Temperature and time of the HAP separation procedure

The first step in the HAP separation procedure consisted of incubation of HAP with the already equilibrated receptor-ligand reaction mixture Simultaneous incubation of HAP with the other constituents to equilibrium proved less appropriate, since the amount of specifically bound ^{125}I-EGF in those incubations was lower than if the HAP suspension was added after equilibration of the receptor preparation with the ligand

This was probably due to shielding of a number of ligand binding sites when HAP was added before equilibrium was reached
After incubation with HAP, the mixture is centrifuged and, after decanting of the supernate, the HAP pellet is washed with assay buffer to eliminate free EGF Interestingly, kinetic dissociation experiments revealed that the net dissociation (i e the overall effect of both dissociation and reassociation) upon the addition of an amount of buffer was faster at 4 °C than at 20 °C (Fig 2 1) The incubation with HAP and the subsequent washing steps were therefore performed at 20 °C
In Fig 2 1 it is shown that a new equilibrium situation was achieved within incubation for 1 h at 20 °C It was of importance to know whether calculation of the binding data had to be performed using the incubation volume before the one hour of incubation with HAP, or using the final volume Therefore, an experiment was performed in which different volumes of HAP suspension, containing the same amounts of HAP, were added to different volumes of incubation reaction mixtures, such that the final volumes were identical (conditions I, II, and III, Table 2 2) The binding data obtained were subsequently analyzed by Scatchard analysis (Scatchard 1949) Using the incubation volume for calculation, the apparent Kd-values obtained in the various incubation conditions decreased obviously with increasing incubation volumes (1 65, 1 28, and 0 61 nM, respectively)

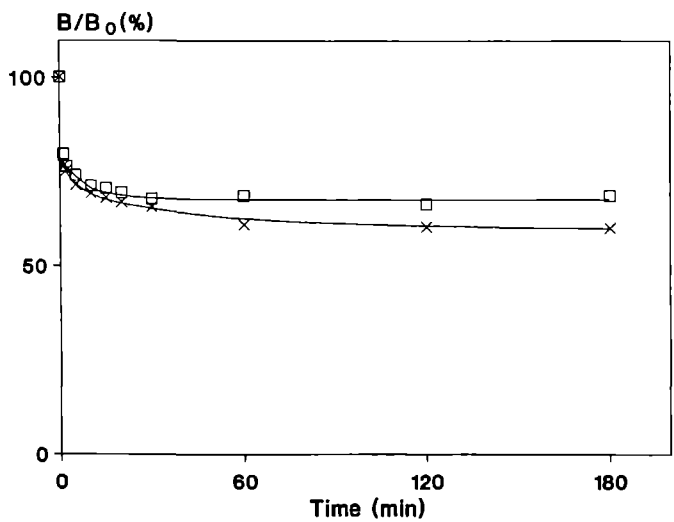

Fig. 2.1 Dissociation kinetics of ^{125}I-EGF
(x), 4 °C, (□), 20 °C HPM was incubated with 3 5 nM ^{125}I-EGF in 140 µl of EGFR assay buffer After incubation for 16 h at 20 °C, 100 µl of HAP suspension was added and incubation was proceeded for another hour at 20 °C The suspension was centrifuged and washed twice with PBB-bufffer Then, dissociation was initiated by the addition of 1 ml of PBB-buffer After various periods of time, the bound fraction was determined by centrifugation of the tube for 2 min at 800 x g, decanting the supernate and counting of the pellet Every point represents the mean of triplicate incubations

Using the final (including HAP) volume for calculation of the results, however, similar Kd's were obtained irrespective of the incubation volume (0.52, 0.70, and 0.47 nM), confirming the establishment of new equilibrium after 1 h of incubation at 20 °C. Therefore, in the ultimate assay procedure the incubation with HAP was performed for 1 h at 20 °C to ensure new equilibrium, and the final volume (incubation volume of membranes with ^{125}I-EGF, and the volume of the added HAP suspension) was used for calculation.

2.3.3 Effect of multiple washing steps on ^{125}I-EGF binding

The HAP incubation was followed by centrifugation and decanting of the supernate containing the nonbound EGF. For optimal elimination of the nonbound EGF it was necessary to perform additional washing steps.

***Table 2.2** Effect of incubation with HAP on ligand-receptor equilibrium.*

	I	II	III
^{125}I-EGF (μl)	10	10	10
PBB-buffer (μl)	30	130	230
HPM (μl)	100	100	100
–> Incubation volume (μl)	140	240	340
HAP volume (μl)	100	100	100
Buffer (μl)	200	100	0
–> Final volume (μl)	440	440	440
Scatchard analysis:			
N (fmol/ml)	292	299	294
$Kd_{inc\ volume}$ (nM)	1.65	1.28	0.61
$Kd_{final\ volume}$ (nM)	0.52	0.70	0.47

HPM was incubated with ^{125}I-EGF (0 1-2 0 nM) in 140, 240 or 340 μl of assay buffer respectively. After incubation for 16 h at 20 °C, identical amounts of HAP in different volumes of assay buffer were added such that the final incubation volumes were identical for all incubations Incubation was proceeded for another hour at 20 °C. Then the tubes were centrifuged for 2 min at 800 x g and the HAP pellets were washed twice with PBB-buffer (20 °C) and subsequently counted for radioactivity Results were calculated using the incubation volume (before the addition of HAP) or using the final volume (after the addition of HAP) N= apparent number of receptors, Kd= apparent dissociation constant

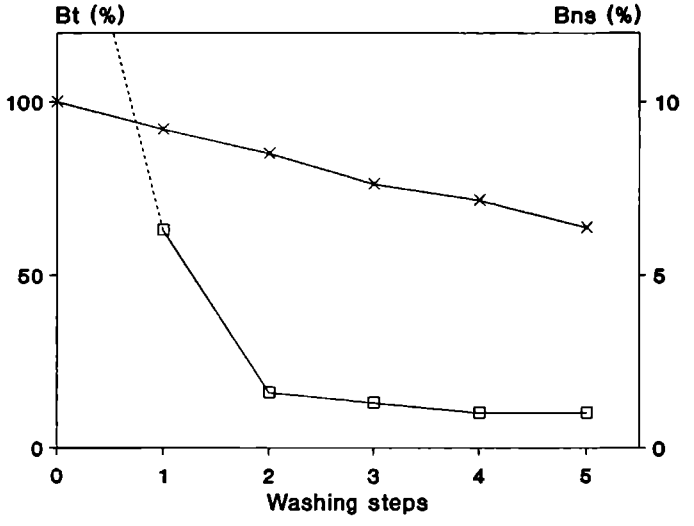

Fig. 2.2 Effect of washing steps on EGF binding.
(x), Bs (=specific binding); (□), Bns (=nonspecific binding). Breast tumor membrane preparations were incubated with ^{125}I-EGF. After incubation with HAP for 1 h at 20 °C, the incubation mixtures were centrifuged for 2 min at 800 x g. The HAP pellets were counted for radioactivity and subsequently washed several (1-5) times with 1 ml of assay buffer without BSA. After every washing step the HAP pellets were counted for radioactivity.

The blank HAP binding experiments indicated that two washing steps were necessary to minimize blank HAP binding as much as possible. In Fig. 2.2 it is shown that nonspecific binding profoundly decreased after the first two washing steps. Subsequent washing steps only marginally decreased nonspecific binding. In Fig. 2.2 it is further shown that the total binding of ^{125}I-EGF decreased slightly after each washing step. Specific binding, the difference between total and nonspecific binding, also decreased slightly after each washing step, leaving approximately 85% of the specifically bound EGF after two washing steps. As a compromise, because of the relatively low nonspecific binding and the relatively high specific binding, it was decided to perform two washing steps with 1 ml of PBB-buffer.
The observed decrease in specific binding by the dissociating washing steps was not due to a release of receptor protein from the HAP, but was exclusively due to dissociation of ^{125}I-EGF from the receptor, which was concluded from the data shown in Fig. 2.3. In this experiment, the reaction mixture after incubation with HAP was centrifuged for 2 min at 800 x g or for 1 h at 105,000 x g. In both cases the radioactivity in the supernates or associated with the HAP pellet were similar (Fig. 2.3).

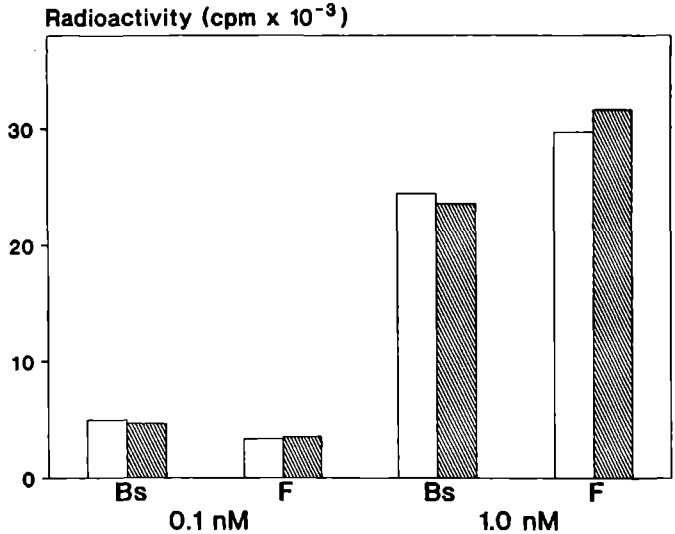

Fig. 2.3 Effect of washing steps: dissociation of radioactivity from HAP after the addition of buffer. HPM was incubated with 0.1 nM or 1.0 nM ^{125}I-EGF for 1 h at 20 °C and 100 µl of HAP suspension was added. After an additional incubation for 1 h at 20 °C, the reaction mixture was centrifuged for 2 min at 800 x g. The HAP pellet was washed once with 1 ml of PBB-buffer and resuspended in 1 ml of PBB-buffer (20 °C). After incubation for 3.5 h at 20 °C, the suspension was centrifuged either for 2 min at 800 x g (empty bars) or for 1 h at 105,000 x g (hatched bars). The pellet and 100 µl of the supernate were counted for radioactivity in a gamma counter.

Moreover, the supernate remaining after the first centrifugation step was again centrifuged for 1 h at 105,000 x g to pellet non-HAP-bound membranes containing EGFR, but no radioactivity could be precipitated.

2.3.4 Binding capacity of HAP

To study the capacity of HAP to bind EGFR-containing membranes, a representative mammary tumor membrane preparation containing 1.0 mg of membrane protein per ml was incubated with 1 nM ^{125}I-EGF. 140 µl aliquots of this incubation mixture (correspondinging to 100 µl of pure membrane preparation, i.e. 100 µg of membrane protein) were added to increasing volumes of HAP suspension. As is shown in Fig. 2.4a, the amount of ^{125}I-EGF specifically bound increased at increasing HAP volumes. A plateau was reached at about 60 µl of HAP, indicating that 60 µl of HAP was sufficient to bind quantitatively the membranes in this experiment. 100 µl of HAP suspension was used for further experiments.

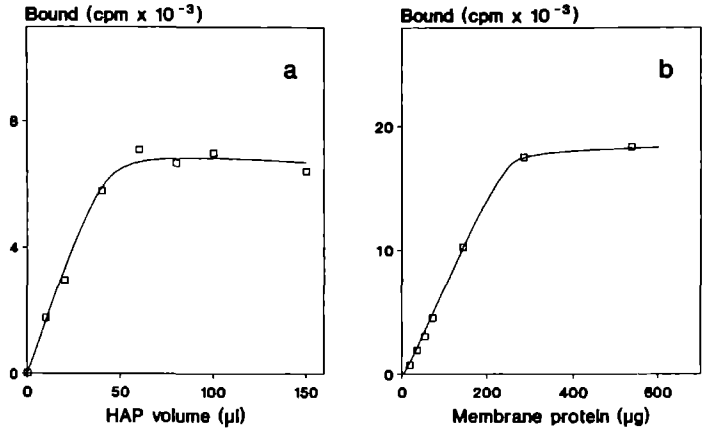

Fig. 2.4 Capacity of HAP
a, Effect of increasing volumes of HAP on the amount of specifically bound ^{125}I-EGF b, Capacity of 100 µl HAP to bind EGFR-containing membranes 5 ml of a tumor membrane preparation (1 0 mg membrane protein/ml) was incubated with 1 0 nM ^{125}I-EGF for 16 h at 20 °C in a final volume of 7 ml Nonspecific binding was assessed in a parallel incubation with 250-fold excess of unlabeled EGF After incubation with HAP, the radioactivity bound was determined after two washing steps with PBB-buffer

Subsequently, increasing amounts of the incubation mixture were added to 100 µl of HAP suspension A linear relationship was observed between the amount of incubation mixture added and the amount of specifically bound ^{125}I-EGF Up to 250 µg of membrane protein could be bound quantitatively to 100 µl of HAP suspension In the ultimate EGFR assay 100 µl aliquots of the samples were intended to be used, so membrane preparations containing protein levels up to 2 5 mg/ml could be assayed using 100 µl of HAP suspension without overflowing the HAP capacity. The ultimate assay was therefore performed with 100 µl of HAP suspension.

2.3.5 Effect of time and temperature on ^{125}I-EGF binding to HPM

Kinetic experiments were performed at 4 °C, 20 °C, 30 °C and 37 °C to investigate the time and temperature required to reach maximum EGF binding to its receptor Fig 2.5 shows that maximum binding was achieved at 20 °C and remained constant for at least up to two days At 30 °C and 37 °C, specific ^{125}I-EGF binding was maximal within 2 h of incubation, but decreased after extended incubation times Experiments in which incubation was performed in the presence of protease inhibitors (tosyl-phenylalanine chloromethylketone, TPCK, 1 mM, and tosyllysyl chloromethylketone, TLCK, 1 mM, Urban *et al.* 1979) showed that the binding of ^{125}I-EGF remained

maximal for at least one day, even at 30 °C and 37 °C (data not shown) This suggests that endogenous proteases were present in the HPM preparation
At 4 °C, the resulting specific binding of ^{125}I-EGF was substantially lower than at the other temperatures examined, even after two days of incubation In the final assay procedure, an incubation period of 16-20 h at 20 °C was used for the measurement of EGFR.

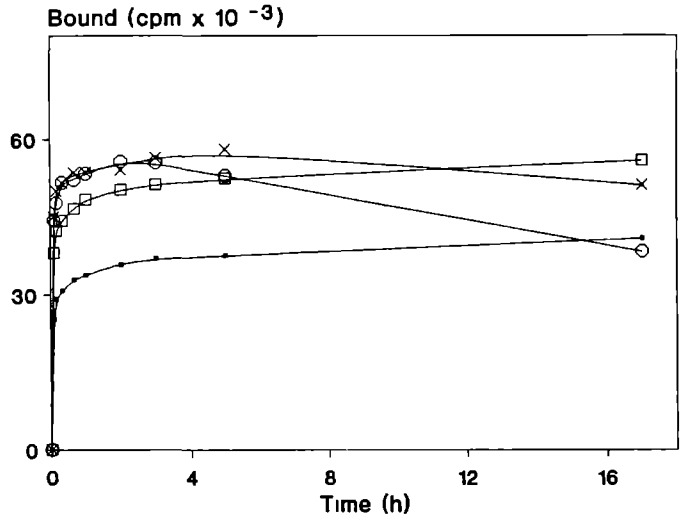

Fig. 2.5 *Association kinetics of EGF binding to HPM at different temperatures (•), 4 °C, (□), 20 °C, (x), 30 °C, (o), 37 °C HPM was diluted 5-fold with assay buffer from a stock HPM (100 µl) and HAP (100 µl) were mixed and pre-incubated for 1 h at 20 °C to be able to study the initial binding kinetics ^{125}I EGF (1 nM) was added at time-point zero and incubated for various periods of time The incubation mixtures were centrifuged for 2 min at 800 x g, the supernates were decanted, and the HAP pellets were rapidly washed twice with 1 ml of assay buffer The resulting HAP pellets were counted for radioactivity to determine bound EGF Nonspecific binding was determined by the addition of ^{125}I-EGF and 250 nM unlabeled EGF simultaneously The data points are taken from one experiment performed in duplicate*

2.3.6 Ligand concentration range

In the ligand binding assay of EGFR described in the present thesis the so-called saturation technique is used (the binding of increasing amount of radioactive ligand). It is assumed that saturation is achieved at the highest concentration of ligand used Analysis of the pattern of binding upon incubation of an HPM preparation with increasing concentrations of ^{125}I-EGF (0 02-7 7 nM) revealed that saturation occurred

at about 2 nM of tracer (Fig. 2.6a). Similarly, analyzing membrane preparations obtained from human breast tumors, saturation of specific EGF binding sites was reached at about 2 nM ^{125}I-EGF (Fig. 2.7a). Accordingly, for routine analysis of EGFR in breast tumor membranes we decided to employ ligand concentrations up to 2.0 nM. The lowest concentration of ligand used in the routine assay of breast tumor samples was 0.1 nM. Ideally, the lowest ligand concentration would be about 0.1 x Kd (Kermode 1989). Assuming a Kd of about 0.5 nM for the high affinity EGFR, this concentration would be 0.05 nM. Practically, at this very low concentration of ^{125}I-EGF specific binding turned out to be too low to be measured significantly, since often low amounts of receptors are present in breast tumor membranes. However, HPM preparations containing high levels of EGFR could be analyzed using ^{125}I-EGF concentrations down to as low as 0.02 nM (vide infra). Due to the low number of EGFR present in the majority of human breast tumor samples, generally a low amount of specific ^{125}I-EGF binding occurs at the lower concentrations of tracer. Therefore, we used a saturation assay (labeled ligand only) in stead of a competitive assay (fixed concentration of labeled ligand and increasing amounts of unlabeled ligand).

2.3.7 Scatchard analysis of the binding data

Scatchard analysis (Scatchard 1949) of the binding data was performed using a nonlinear weighted regression analysis employing Marquardt's algorithm (Marquardt 1963), which fits the experimental binding data into either a one binding site model or a two binding site model (see addendum). Chi-square analysis gives the opportunity to discriminate between the two possible models. Scatchard plot analysis performed on the binding data obtained with the HPM showed a curvilinear pattern as is shown in Fig. 2.6b. Analysis of these binding data according to the two binding site model revealed the presence of a binding moiety with an extremely high affinity, Kd 0.01 nM, and one with a Kd of 0.32 nM, which is generally regarded as the high affinity EGFR. If such binding data were evaluated according to the one binding site model, the apparent ligand binding parameters obtained depended on the concentration range of EGF used. The higher the concentration range, the higher EGFR values and apparent Kd's assessed (Table 2.3).
Scatchard plots derived from the binding data obtained with breast tumor membranes (Fig. 2.7b), however, showed the best fit according to the one binding site model, even in those cases in which slightly curvilinear Scatchard plots were observed.
So far, in the handling of the receptor binding data an experimentally estimated nonspecific binding was used (250-fold excess of unlabeled EGF) and the residual binding of ^{125}I-EGF was then subtracted from the experimental data (Rosenthal, 1967). In the one binding site model, used for the calculation of the binding data, it was also possible to use a computer estimated value of the nonspecific binding, assuming nonsaturable behaviour only within the range of ligand concentrations tested. However, for the individual tumors analyzed the use of this computer estimated nonspecific binding did not result in a significant improvement of the binding data, as derived from chi-square analysis. It was therefore decided to use the one binding site model employing the experimental estimate for nonspecific binding.

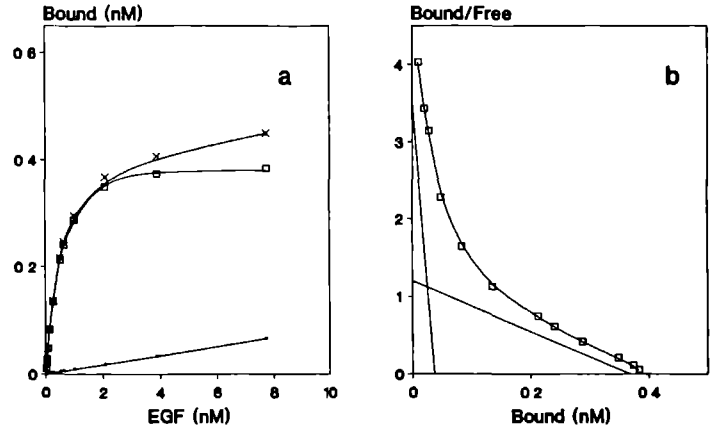

Fig. 2.6 a, Binding of ^{125}I-EGF following incubation of increasing concentrations of ^{125}I-EGF (0 02-7 7 nM) to HPM, (x), total binding, (•), nonspecific binding obtained with a 250-fold excess of unlabeled EGF, (□), specific binding b, Scatchard plot of the specific binding data from Fig 2 6a.

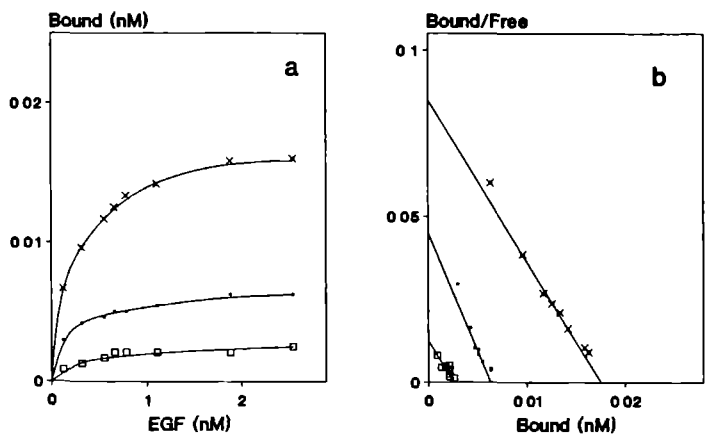

Fig. 2.7 a, Specific binding of ^{125}I-EGF following incubation of increasing concentrations of ^{125}I-EGF with three human breast tumor membrane preparations b, Scatchard plots of the binding data from Fig 2 7a.

***Table 2.3** Effect of the ligand concentration range on the apparent binding parameters*

Range (nM)	EGFR (pM)	Kd (nM)
0.01-0.5	707	0.12
0.01-1.0	823	0.16
0.02-2.0	901	0.20
0.04-3.9	930	0.22
0.07-7.7	939	0.23

The ^{125}I-EGF binding data obtained with HPM were analyzed with the one binding site model, employing an experimental estimate for nonspecific binding (measured by using a 250-fold excess of unlabeled EGF).

2.3.7 Ultimate EGFR assay procedure

The data presented in the previous paragraphs have led to the ultimate EGFR assay procedure. A flowchart of this procedure for the assessment of EGFR in membrane preparations is shown in Table 2.4.
Eight 100 µl aliquots of membrane fraction were incubated in polystyrene assay tubes with increasing concentrations of ^{125}I-EGF in a final volume of 140 µl of EGFR assay buffer (20 mM phosphate, 150 mM NaCl, 50 µM bacitracin, 0.1% BSA, pH 7.4) for 16-20 h at 20 °C.
The concentrations of ^{125}I-EGF in the incubation mixtures ranged from 0.15 nM to 3.3 nM. Nonspecific binding was assessed in parallel incubations (in duplicate) in the presence of 1.0 nM ^{125}I-EGF and 250 nM unlabeled EGF.
For separation of receptor-bound from free EGF, 100 µl of HAP suspension was added to each tube. The final concentrations of ^{125}I-EGF ranged from 0.1 nM to 2.0 nM.
The tubes were incubated for another hour at 20 °C to ensure binding of EGFR to HAP and to reach a new equilibrium. During this incubation the tubes were vortex-mixed 3-4 times.
After centrifugation for 2 min at 800 x g (20 °C) the supernates, containing the unbound ligand, were decanted.
The pellets were washed twice with 1 ml of PBB-buffer (20 °C) and then centrifuged for 2 min at 800 x g (20 °C).
Finally the radioactivity associated with the HAP pellet was counted in a gamma counter.
Calculation of the results by Scatchard analysis was performed using the nonlinear weighted regression curve fitting program according to the one binding site model, using 240 µl as incubation volume and with the experimentally estimated nonspecific binding.

Table 2.4 Flowchart for the measurement of EGFR in membrane preparations

	Tubes 1-8	Tubes 9-10
^{125}I-EGF (µl)	10	10
Unlabeled EGF (µl)	-	10
EGFR assay buffer (µl)	30	20
Membrane preparation (µl)	100	100
Incubate for 16-20 h at 20 °C		
HAP suspension (µl)	100	100
Incubate for 1 h at 20 °C		
Centrifuge for 2 min at 800 x g		
Decant supernate and add 1 ml of EGFR assay buffer without BSA		
Vortex and centrifuge for 2 min at 800 x g		
Decant supernate and repeat washing step		
Count final HAP pellet in gamma counter		
Calculation of results using 240 µl incubation volume		

2.4 Discussion

The assessment of EGFR in human breast cancer biopsies is generally performed using a biochemical radioligand binding assay. As was shown in Table 2.1, research groups reporting the assessment of EGFR all employ different assay conditions. This may provide an explanation for the large variation in EGFR positivity and EGFR levels and Kd's that have been reported.

Different procedures for the isolation of the cell membranes have been shown to yield different EGFR levels (Dittadi *et al.* 1990b). In view of standardization of the assay, we were restricted to use the crude membrane fraction that is obtained during preparation of tumor cytosols (100,000 x g - 800 x g) for routine steroid hormone receptor analysis according to EORTC guidelines (EORTC Breast Cancer Cooperative Group 1980). The majority of studies included in Table 2.1 obey to this guideline an therefore the membrane isolation procedure we used does not differ widely.

In most of the studies, the preparation of radioiodinated EGF (^{125}I-EGF) was performed by using Chloramine T or Iodogen. It may be emphasized that these are relatively harsh reagents (Bolton 1985, chapter 4 of this thesis), resulting in oxidation and aggregation of iodinated peptide molecules. Moreover, EGF contains five tyrosine residues (Savage *et al.* 1972) sensitive to iodine incorporation, while some of these may be involved in receptor binding (Engler *et al.* 1990,1991, Campion *et al.* 1991).

Although receptor affinity and capacity estimates can be significantly affected when there is no equivalence in binding behavior between labeled and unlabeled ligand (Taylor 1975, Hollemans and Bertina 1975, Kermode 1988), investigators rarely test whether iodinated ligands behave the same as the natural ligand in receptor binding (Kermode 1988). In the case of ^{125}I-EGF, no thorough attempts have been made to determine the binding behavior of this radioligand relative to that of unlabeled EGF. A study of Nicholson *et al.* (Nicholson *et al.* 1988) forms an exception. In that study it is reported that in their so-called displacement assay (using a fixed concentration of ^{125}I-EGF and varying amounts of unlabeled EGF) the Kd's observed were significantly higher (the affinity lower) than in their so-called saturation technique (using increasing concentrations of ^{125}I-EGF).

In the various reports on the assessment of EGFR in breast tumor membranes, different concentration ranges of ^{125}I-EGF have been used in multiple point titration assays as well as different concentrations in single dose analyses, as was shown in Table 2.1. These differences in ligand concentrations in single point assays certainly have led to substantial differences in EGFR results. As a matter of fact, the level of saturation of the receptor not only depends on the concentration of the ligand used, but also on the affinity of the receptor for the ligand. If in a tumor only one class of binding sites is present, e.g. with a Kd of 0.3 nM, a single point assay using a tracer concentration of 0.6 nM would saturate about 70% of the receptors. In another tumor with receptors with a Kd of 0.6 nM, the single point assay with the same tracer concentration would only result in a calculated receptor concentration of 50% of the receptors present. Results obtained in studies employing different concentrations of ligand therefore hardly can be compared with each other.

In multipoint assays different ligand concentration ranges yield different EGFR values and apparent Kd's if curvilinear Scatchard plots are obtained. This phenomenon is represented by the results of the experiment collected in Table 2.3. In our ligand binding assay we used the saturation technique in stead of the competition technique. The concentrations of ^{125}I-EGF used in the assay ranged from 0.1 nM to 2.0 nM. The upper concentration of 2.0 nM was employed since it appeared that saturation of specific binding sites occurred at that concentration. The lower concentration of ^{125}I-EGF used ideally would have been about 0.1 x Kd (Kermode 1989), which corresponds to about 0.05 nM assuming a Kd of about 0.5 nM. At this very low concentration of ^{125}I-EGF the specific counts bound become too low. Accordingly, we used 0.1 nM ^{125}I-EGF as the lowest concentration of tracer. Since the binding data obtained with breast tumor membranes showed the best fit according to the one binding site model, despite the slightly curvilinear Scatchard plots that were observed in some experiments, we used this one binding site model including the actual measured nonspecific binding for calculation of the binding data routinely. The slight curvilinearity of some of the Scatchard plots suggest a heterogeneous receptor population as will be discussed in more detail in chapter 7.

As far as the estimate of nonspecific binding is concerned, it has been reported that nonspecific binding may in fact be saturable at very high ligand concentrations (Mendel and Mendel 1985). Therefore, the concentration of unlabeled EGF sufficient to eliminate all specific binding of labeled EGF is uncertain. Therefore, methods have been developed to determine ligand binding parameters without measuring nonspecific binding (Munson and Rodbard 1980, Van Zoelen 1989, see also addendum). For the individual tumors analyzed in our studies the binding data

obtained using this computer-fitted value for nonspecific binding were not significantly improved as compared to those obtained by direct subtraction of experimentally measured nonspecific binding. While the excess of unlabeled EGF used (150 nM) is sufficient (250-350 x Kd) to block >99% of the receptors, a larger excess may cause saturation of nonspecific sites or very low affinity binding sites yielding underestimated nonspecific binding values, and is therefore considered not appropriate (Chamness and McGuire 1975).

According to the time and temperature of the equilibration step in EGFR assays, we found an incubation period of 16 h at 20 °C to be most favorable, although various investigators employ incubation conditions of one or two hours at room temperature. Such short incubation times may however be sufficient in case of employing a single point assay at a high concentration of ligand, but certainly not for the lower concentrations of ligand in the multiple point titration assays.

Last but not least, different procedures for the separation of receptor-bound from free ligand have been reported. Achieving an optimal procedure for the separation of receptor-bound from free ligand is a serious challenge in receptor binding studies. Often, (ultra) centrifugation and (ultra) filtration are used (Table 2.1), methods which are probably adequate in the case of whole cell assays, or EGFR assays on other relatively large particles such as vesicles (Ensing *et al.* 1986, Berkers *et al.* 1990). In the case of small membrane fragments, quantitative pelletting of receptor-ligand complexes is only possible when the speed of centrifugation is very high (Dittadi *et al.* 1990b). This is however a relatively time-consuming procedure, needs sophisticated equipment and cannot be performed on a large scale routine basis. Moreover, equilibrium conditions are easily perturbed using these separation methods (Ensing *et al.* 1986), which can lead to a substantial misinterpretation of the binding data (Munson 1983). We introduced HAP to overcome the difficulties in achieving quantitative pelletting of the membranes. HAP was found to quantitatively bind membranes containing EGFR, leaving the nonbound ^{125}I-EGF in solution (Benraad and Foekens, 1990). Only low-speed centrifugation is subsequently required for the separation step, which can therefore be performed rapidly with minimal dissociation of ligand-receptor complexes. The binding capacity of the routinely used HAP suspension was sufficient to analyze membrane fractions with a membrane protein content less than 2.5 mg/ml, which is the case in nearly all of the tumor membrane preparations.

To optimize the HAP separation procedure several assay conditions were evaluated. Assay sensitivity increased markedly if BSA and bacitracin was included in the assay buffer, since the blank HAP binding of ^{125}I-EGF, and therefore also nonspecific binding, was diminished. The bacitracin molecules likely preclude the binding of EGF to the HAP mineral. A very small amount of the ^{125}I-EGF remained bound to HAP, even after multiple washing steps. As stated above, dissociation of receptor-ligand complexes has to be avoided as much as possible. Washing of the HAP pellet is however unavoidable since otherwise the bound fraction will be disturbingly contaminated with free ligand, which also affects the outcome of the receptor assay (Munson 1983). Actually, the most favorable assay conditions were chosen as a compromise between specific binding, nonspecific binding, and dissociation of ligand from the receptor. Regarding the latter, the HAP washing steps could be performed most conveniently at room temperature, since the net dissociation (i.e. the overall effect of both dissociation and reassociation) at 20 °C was less as compared to 4 °C.

It became evident that the reassociation of EGF at 20 °C was faster than at 4 °C (Fig. 2.5). It is worth mentioning that this phenomenon has not been taken into account by the investigators that employ a high-speed centrifugation procedure for the bound-free separation step. In those studies, an aliquot of ice-cold buffer is added prior to the centrifugation step.

In summary, an assay method involving HAP is described for the measurement of EGFR in cell membrane preparations. Separation of the receptor-ligand complexes from free ligand by HAP is rapid, such that dissociation of ligand-receptor complexes is relatively small. Moreover, only low-speed centrifugation is required for the separation step such that large series of samples can be analyzed simultaneously. The present assay procedure is easily applicable to the pellet fraction which remains after standard preparation of cytosols for routine assay of steroid hormone receptors according to EORTC guidelines. The methodology described will enable every laboratory which is currently involved in measurement of steroid hormone receptors to introduce an EGFR assay.

References are listed on pages 99-110.

CHAPTER 3

EVALUATION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR HYDROXYLAPATITE ASSAY

3.1 Introduction

Hydroxylapatite (HAP) was introduced in the EGFR radioligand binding assay for the separation of epidermal growth factor receptor-bound from free ^{125}I-EGF (chapter 2). Only low-speed centrifugation is subsequently required for separation of bound from free ligand. Experimental data on the actual performance of the HAP assay are presented in this chapter. The HAP assay was compared with the generally performed centrifugation- and filtration assays, using human placental membranes (HPM) and mammary tumor membranes. Moreover, in view of the potential importance of the EGFR status of primary breast tumors, the suitability of the HAP assay to act as a standard EGFR assay method was tested in a pilot interlaboratory quality control trial.

3.2 Methods

3.2.1 General

Human placental membranes (HPM) and human breast tumor membranes were obtained as described in the previous chapter. EGF was radioiodinated by using either Protag-125, iodine monochloride (ICl), or Enzymobeads to a specific radioactivity of about 500 Ci/mmol (see chapter 4 for details). Human recombinant EGF (hEGF) was obtained from Bioproducts for Science Inc. (Indianapolis, IN, U.S.A.). Human recombinant transforming growth factor-α (hTGFα) was obtained from Peprotech Inc. (Rocky Hill, NJ, U.S.A.).

3.2.2 EGFR assays

The HAP procedure for the measurement of EGFR was performed as described in the previous chapter.
In the centrifugation assay, 1 ml of PBB-buffer (20 mM phosphate, 150 mM NaCl, 50 μM bacitracin, pH 7.4) was added to the equilibrated reaction mixtures (20 °C) and centrifugation was performed for 15 min at 100,000 x g. Subsequently, the supernates were aspirated and the pellets were counted for radioactivity.
The ultrafiltration procedure was performed by pipetting 100 μl of the incubation mixture onto a Whatman GF-C filter (Whatman International Ltd., Maidstone, U.K.) that was placed in a microfiltration unit. The filters were washed twice with 5 ml of PBB-buffer (20 °C), after which the filters were counted for radioactivity.
Further details of the centrifugation and of the filtration assays were identical to those of the HAP assay.

3.2.3 Preparation of quality control samples

Membranes obtained from human placenta were diluted with appropriate volumes EGFR assay buffer to yield EGFR concentrations of about 40 and 80 fmol/ml respectively. Two-milliliter aliquots of the resulting preparations were lyophilized. The vials were sealed under vacuum and stored at 4 °C until shipment by regular mail. The

samples were shipped to ten EORTC laboratories. These laboratories received 4 vials (two of each concentration) of lyophilized control samples per trial, and also a lyophilized ^{125}I-EGF preparation. Samples were reconstituted with 2 ml of water and analyzed for EGFR.

3.3 Results

3.3.1 Reproducibility

In several independent experiments performed in our laboratory, the reliability of the HAP assay was determined. The results of the examination of the within- and between-assay variation of the HAP assay using HPM preparations that contained different amounts of EGFR are depicted in Table 3.1. The within-assay coefficient of variation of 8 individual experiments was found not to exceed 10%, even for EGFR levels down to as low as 4.1 fmol/ml. The between-assay variation, using different batches of ^{125}I-EGF, was found to be about 13%, and the between-assay variation using one single batch of ^{125}I-EGF was 8.6% (Table 3.1).

***Table 3.1** Reproducibility of the HAP assay assaying HPM*

	n[1]	EGFR mean ± S.D. (fmol/ml)	C.V.[2] (%)
Within-assay variation	6	4.1 ± 0.3	6.6
	6	6.4 ± 0.5	8.5
	6	11.6 ± 0.7	5.8
	7	120 ± 4	3.2
	10	159 ± 15	9.5
	12	173 ± 9	5.2
	4	203 ± 12	5.8
	15	208 ± 15	7.2
Between-assay[3] variation	8	122 ± 15	12.6
	42	184 ± 25	13.5
	5	418 ± 36	8.6

[1] *number of determinations*
[2] *coefficient of variation.*
[3] *The between-assay variation data in the upper two rows were obtained with several batches of ^{125}I-EGF (between-batch between-assay variation), whereas the lower row was obtained with a single batch of ^{125}I-EGF (within-batch between-assay variation).*

The assay sensitivity was evaluated by experiments in which serial dilutions of HPM were prepared in EGFR assay buffer to yield membrane preparations containing various concentrations of EGFR. The results of one representative experiment are depicted in Fig. 3.1. It is shown that the EGFR concentration in the diluted HPM samples correlated well with the corresponding dilution factor.
The lower limit of sensitivity was defined as the lowest concentration of EGFR present in the membrane preparation that could be measured reliably. The lowest EGFR concentration that could be measured reliably was about 3 fmol/ml. Human breast tumor membranes generally show a somewhat higher nonspecific binding as compared to the HPM preparation used. If nonspecific binding accounts for a substantial amount of the total binding, the specific binding may be masked by the nonspecific counterpart, which can result in false negative EGFR concentrations. Actually, the lowest concentration of EGFR measured in a human breast tumor cell membrane preparation was 3 fmol/ml.

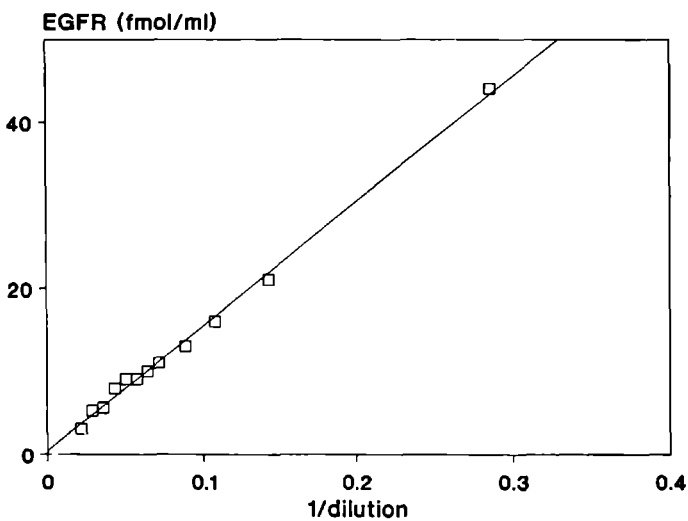

***Fig. 3.1** EGFR in serially diluted HPM.*
An HPM preparation was diluted with assay buffer. The membrane preparations obtained were analyzed for EGFR by the HAP assay.

3.3.3 Recovery of EGFR

An "EGFR recovery experiment" was performed to investigate the possible interference of nonspecific proteins present in tumor membranes on the outcome of the HAP assay. 10 µl aliquots of HPM, which contained 90 and 1136 fmol EGFR respectively, were added to human breast tumor biopsy membrane preparations with different concentrations of membrane protein (and EGFR). The apparent number of receptors subsequently measured by the HAP assay (recovery) was similar to that expected by calculation of the sum of the respective amounts of EGFR in the membrane fractions (Table 3.2). In this experiment the apparent Kd's measured after the addition of the tumor membranes did not differ from those measured before this addition (0.59 ± 0.30 nM and 0.69 ± 0.18 nM respectively).

***Table 3.2** Recovery of EGFR*

Number of membrane preparations	Tumor EGFR present (fmol/ml)	HPM EGFR added (fmol)	Recovery (%) (mean ± S.D.)
26	0	1136	103 ± 11
19	0	90	98 ± 11
1	9	90	93
1	47	90	98
1	19	90	103
1	70	90	110
1	8	90	106
1	623	90	98
1	5	90	112

10 µl of HPM preparation containing 90 and 1136 fmoles of EGFR protein respectively were added to 100 µl of human breast tumor membranes containing EGFR/ml as indicated. The recovery was calculated as the percentage of EGFR relative to the expected value (the sum of the respective amounts of EGFR).

3.3.4 Is occupied EGFR measured in the assay?

Human breast tumors produce EGFR ligands (EGF, TGFα) (Dickson and Lippman 1987). Furthermore, EGFR ligand(s) have been detected in the cytosols of human primary breast tumors (Macias *et al.* 1989, Foekens *et al.* 1989b). Membrane preparations derived from such tumors may also contain these ligands, either or not complexed to the receptor. Therefore, an experiment was performed to disclose whether also EGFR occupied by its ligands is measured under the conditions used. In other words, do endogenous ligands mask more or less the presence of EGFR.

***Table 3.3** Effect of the addition of EGF and TGFα to HPM*

membranes:		not centrifuged		centrifuged	
		EGFR (fmol/ml)	Kd (fmol/ml)	EGFR (fmol/ml)	Kd (fmol/ml)
hEGF (nM)	0.0	361	0.32	353	0.31
	0.1	368	0.31	334	0.33
	1.0	390	0.60	326	0.44
	2.0	391	0.90	330	0.63
	10.0	358	3.05	346	0.54
hTGFα (nM)	0.0	361	0.30	353	0.31
	0.1	341	0.32	343	0.32
	1.0	369	0.51	358	0.37
	2.0	366	0.63	352	0.37
	10.0	376	2.18	358	0.37

A HPM preparation was incubated with the indicated concentrations of hEGF or hTGFα in EGFR assay buffer for 2 h at 20 °C. EGFR was assessed in this suspension (not centrifuged) and, after centrifugation for 1 h at 100,000 x g, in the membrane pellet (centrifuged). Calculation of the results was performed using the specific radioactivity of the tracer.

An HPM suspension was preincubated with different concentrations (up to 10 nM) of hEGF or hTGFα. EGFR was assessed in the suspension and also, after centrifugation, in the membrane pellet. In the latter procedure the presence of receptors occupied with endogenous ligands is mimicked. The data obtained in this experiment are collected in Table 3.3.
Without centrifugation, irrespective of the addition of ligands, the multiple point HAP assay resulted in similar EGFR levels, even at 10 nM hEGF or hTGFα. These results demonstrate a reversible interaction between EGFR and the ligands added. Furthermore it appeared that the addition of 1 nM of ligands resulted in a nearly two-fold increase of the apparent Kd, and the addition of 10 nM of ligands in a seven- to ten-fold increase of the apparent Kd.
In the experiments in which the suspension was centrifuged all membrane preparations contained similar receptor values and nearly identical Kd's. These results imply that during the membrane isolation procedure at least most of the endogenously present EGFR ligands are eliminated from the membranes and that no interference will be encountered from ligands that are present in the membrane sample.
It is noteworthy that recently a method has been described in which occupied and nonoccupied EGFR was measured in membranes from human breast tumor biopsies, involving acid (glycine pH 3) dissociation of endogenously bound ligand molecules to obviate the possibility of interference by endogenously bound ligands (Falette *et al.*

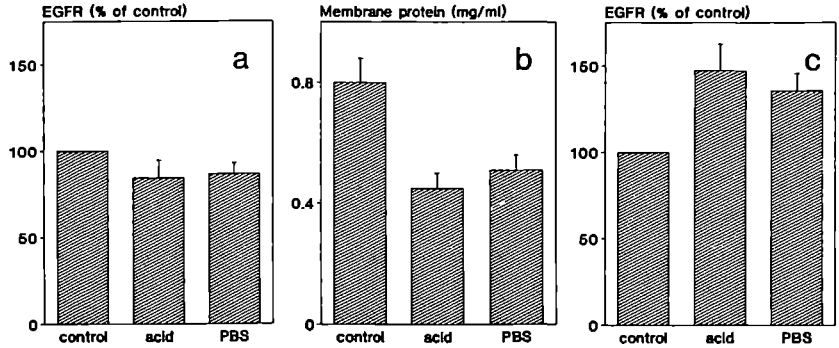

***Fig. 3.2** Mean EGFR and protein levels in tumors upon acid treatment and membrane washing. a, EGFR levels (expressed as fmol/ml), calculated as percentage of control; b, protein concentration (mg/ml); c, EGFR expressed as fmol/mg of membrane protein, calculated as percentage of control.*

1992). In that study, a significant increase in EGFR levels in the tumors was found, as well as an increase in the number of EGFR positive tumors after the acid treatment. Unfortunately however, an essential control experiment was lacking. The addition of buffer (pH 7.4) in stead of acid should have been included in the experiment. Verifying those results, we analyzed mouse and human mammary tumors exactly according to the described method (Falette *et al.* 1992). We included an additional control experiment in which the membrane preparations were washed with neutral EGFR assay buffer (membrane washing) in stead of glycine buffer (acid treatment) to investigate the effect of the membrane processing per se on the EGFR concentration. Mean EGFR concentrations (expressed as fmol/ml) slightly decreased (in stead of increased) upon both acid treatment and membrane washing (Fig. 3.2a) as compared to the untreated membrane preparations. No differences in apparent Kd's could be observed. However, both acid treatment and membrane washing resulted in a significant decrease of the protein concentration in the membrane preparations, when compared to the untreated membrane preparations (Fig. 3.2b, $P=0.0001$ for both acid treatment and membrane washing, paired t-test). Consequently, if EGFR in the membrane preparations was expressed as fmol/mg of protein, like reported by Falette *et al.* (1992), acid treatment and membrane washing resulted in an increase in EGFR levels (Fig. 3.2c). These results suggest that the acid treatment in the radioligand binding assay results in an EGFR-enriched membrane preparation probably by elimination of contaminating cytosol proteins, which however also occurs by membrane washing. So we did not observe any increase in EGFR levels as a result of dissociation of ligands by acid treatment.

3 3 5 Effect of membrane protein on the level of EGFR

Available primary breast tumors assessed for EGFR varied in size Therefore the relation between membrane protein and EGFR levels was studied. Statistical analysis, as described in the thesis by P G Koenders, showed the number of EGFR positives of a total of 725 breast tumor membrane preparations to be positively related to membrane protein levels, with declining percentages of EGFR positives at the lower (<0 2 mg/ml) membrane protein level (Fig 3 3a) As a consequence, a negative relationship was observed between the EGFR levels and the membrane protein levels (Fig 3 3b) This result indicated that false negative assay results easily occur at the lower membrane protein levels When the EGFR estimations obtained with membrane protein levels up to 0 2 mg/ml were disregarded, EGFR levels and membrane protein levels were no longer associated A membrane protein level of 0 2 mg/ml was adopted as a threshold.

3.3.6 Effect of storage of membrane preparations on EGFR

Analysis of the 531 remaining sets of data (membrane preparations with protein levels >0 2 mg/ml) revealed that 57% (302 samples) of the tumor cell membrane preparations contained specific, saturable, high-affinity binding sites for EGF with a mean Kd of 0 5 nM (range 0 1-1 1 nM, 95% of the observations) Total EGFR levels ranged from 3 to 3600 fmol/mg of membrane protein, with a median value of 40 fmol/mg of membrane protein Membrane preparations were stored under an aliquot of PBB-buffer at -80 °C for various periods of time No relationship was found between the percentage of EGFR positives and duration of storage at -80°C, ranging from 1 wk to 3 yr (Fig 3.4a), or between EGFR levels and duration of storage (Fig. 3 4b)

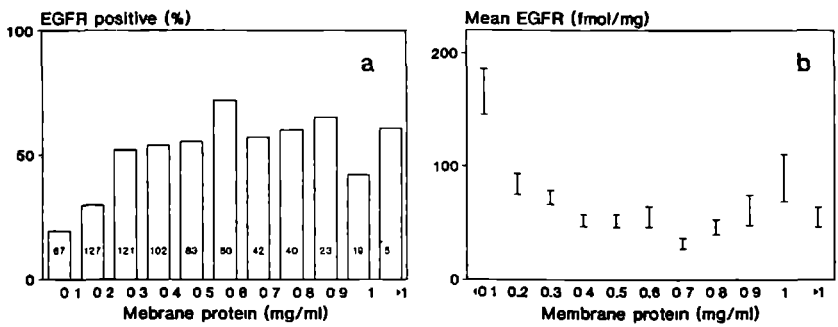

Fig. 3.3 a, Relation between membrane protein concentration and the percentages of EGFR positivity (bars representing percentage of numbers displayed in the bars) b, Relation between membrane protein concentration and EGFR levels Reproduced with permission

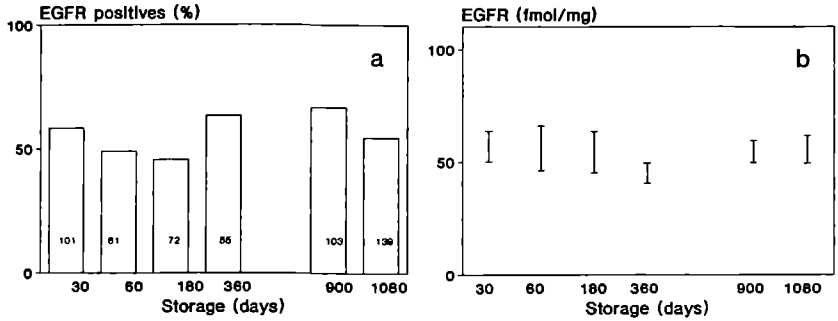

***Fig. 3.4** a, Relation between the duration of storage of the membrane preparations (days), and percentages of EGFR positivity (bars representing percentage of numbers displayed in the bars). b, Relation between duration of storage and mean EGFR concentration of positives (fmol/mg of membrane protein ± SEM). Reproduced with permission.*

3.3.7 Comparison of the HAP bound-free separation procedure with centrifugation and filtration procedures

To study the performance of the HAP assay as compared to the generally used centrifugation and filtration assays, HPM was serially diluted with EGFR assay buffer to yield membrane preparations with six different concentrations of EGFR. Using the HAP assay, EGFR concentrations in the membrane preparations obtained were found to parallel the dilution factor, even if the EGFR concentration in membranes was diluted 32 times resulting in an EGFR concentration of 4 fmol/ml (Fig. 3.5). In both the centrifugation and filtration assay this parallellism was inferior. The larger error bars illustrate the higher variations observed in the centrifugation and filtration assays as compared to the HAP assay (Fig. 3.5b and c, compared to Fig. 3.5a). EGFR concentrations obtained in the centrifugation assay were generally lower than those obtained in the HAP assay ($P<0.0001$, paired t-test) or in the filtration assay ($P=0.003$). The centrifugation assay resulted in a significantly higher apparent Kd (0.29 ± 0.18 nM) as compared to the HAP assay (0.16 ± 0.06 nM) or the filtration assay (0.21 ± 0.12 nM) ($P=0.0001$ and $P=0.03$, respectively), indicating a greater extent of dissociation in the centrifugation assay.
An additional experiment was performed to obtain information regarding the reproducibility of the three assays. A diluted HPM preparation was analyzed for EGFR in ten-fold. As shown in Table 3.5, the within-assay variation of filtration assay was remarkably higher as compared to either the HAP asssay or the centrifugation assay. Scatchard analysis of the receptor binding data obtained in the three respective

assays clearly illustrated the performance of the assays (Fig. 3.6). The data points obtained in the HAP assay were were much less scattering than those in other assays. Particularly at the most diluted HPM, the scattering of the data points in the centrifugation and filtration assays was considerable. Typical examples of the Scatchard plots obtained in the three different assays are shown in Fig. 3.6.
Nonspecific binding in the HAP assay was 1.5-2.3% of the total ^{125}I-EGF added, whereas in the centrifugation and filtration assay 0.8-1.6% and 0.8-2.2% nonspecific binding was observed.

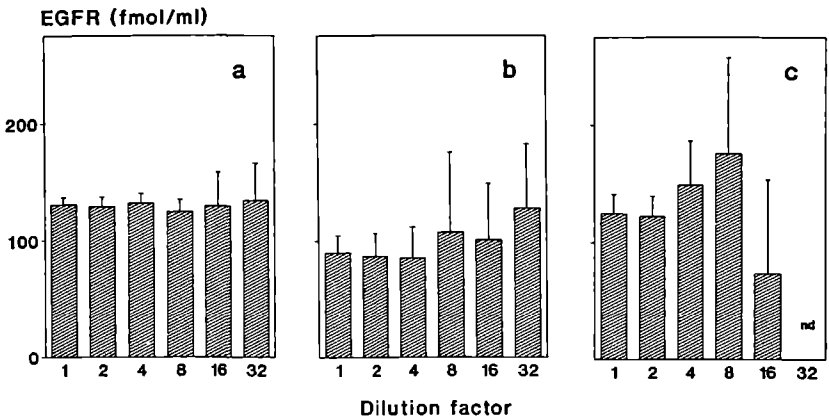

Fig. 3.5 EGFR concentrations in serially diluted HPM measured by using a, the HAP assay; b, the centrifugation assay; c, the filtration assay. EGFR levels were corrected for the respective dilution factor.

***Table 3.5** Within-assay variation of the HAP, centrifugation, and filtration assay.*

Assay	EGFR (fmol/ml)
HAP	13.8 ± 1.9 (14.0%)
Centrifugation	10.6 ± 3.1 (29.3%)
Filtration	4.9 ± 6.2 (126.5%)

A 1000-fold diluted HPM preparation was assessed for EGFR in ten-fold. Numbers represent mean EGFR values ± S.D, n=10. (%= coefficient of variation).

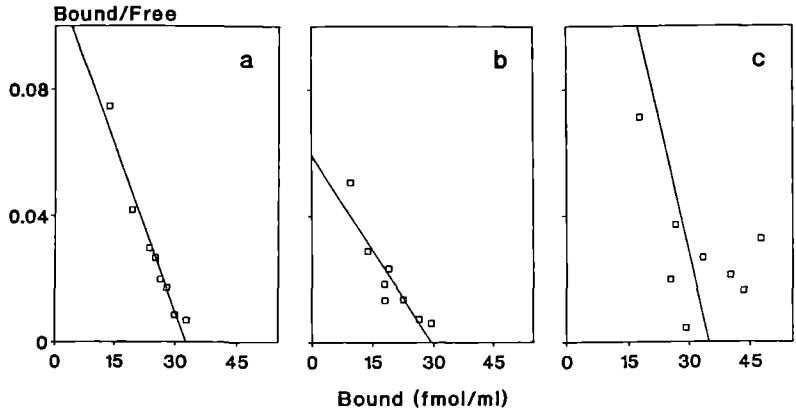

Fig. 3.6 Typical Scatchard plots, a, HAP assay; b, centrifugation assay; c, filtration assay.

Membrane preparations obtained from human, mice and dog mammary tumors were analyzed for EGFR using the three different procedures. The results of these experiments are collected in Table 3.6. The EGFR levels obtained in the centrifugation or filtration assay were expressed relative to the EGFR levels obtained in the HAP assay. Using a multiple point assay in this experiment no significant differences in the apparent numbers of receptors were observed between the HAP, the centrifugation and the filtration assay. However, the apparent Kd's obtained in the centrifugation assay were substantially higher than those obtained in the filtration assay and in the HAP assay. The Scatchard plots obtained in the different procedures were comparable to those shown in Fig. 3.6.

In case EGFR levels were calculated by using a single concentration of ^{125}I-EGF (2 nM), they were lower as compared to the multiple point assay (Table 3.6). The single point HAP assay resulted in higher receptor levels as compared to the centrifugation assay ($P=0.02$). No differences between the HAP assay and the filtration assay nor between the centrifugation assay and the filtration assay could be observed, due to the large variation in assay results in the filtration assay.

Nonspecific binding in the HAP assay ranged from 1.4-3.0% of the total radioactivity added, whereas nonspecific binding in the centrifugation and filtration assays was 0.9-2.9% and 1.4-4.4% respectively.

Table 3.6 *Comparison of EGFR levels obtained by HAP-, centrifugation-, and filtration assays in human, mice, and dog mammary tumor membranes*

Assay	EGFR[1]	EGFR[2]	Kd (nM)
HAP	100 ± 6	78 ± 4	1.03 ± 0.29
Centrifugation	98 ± 14	62 ± 4	3.24 ± 0.79
Filtration	108 ± 60	76 ± 29	1.39 ± 0.56

[1]EGFR measured by multiple point Scatchard analysis
[2]EGFR measured by single point analysis with 2.0 nM ^{125}I-EGF.
EGFR levels obtained with the multiple point HAP assay were set at 100%. The standard deviation represents the mean standard deviation of the experiments in duplicate.

3.3.8 Interlaboratory quality control

The between-laboratory variation of the HAP assay was evaluated in two pilot quality control trials. Hence, a reference membrane preparation had to be prepared which could be transported within Europe at ambient temperature, enabling evaluation of the performance of the HAP assay. Human placental membrane samples were found to contain sufficiently high EGFR levels.
Lyophilized HPM was stored at 4 °C or at room temperature for various periods of time and analyzed for EGFR content. Approximately 60% of the original EGFR activity remained detectable after lyophilization of the samples in the presence of 0.1% BSA, the Kd's being unchanged (0.56 ± 0.16 nM before lyophilization and 0.59 ± 0.18 nM after lyophilization respectively). Omission of BSA resulted in an additional 10% loss (data not shown). Recently performed experiments revealed that lyophilization of HPM in the presence of 10% saccharose resulted in a loss of only 15% of EGFR and did not affect the apparent Kd.
Storage of lyophilized HPM for up to 16 weeks at 4 °C did not result in a significant decrease in measurable EGFR levels, nor did storage for 3 weeks at ambient temperature (Table 3.7). Therefore, it was concluded that lyophilized HPM could be used as reference material in quality control assessments. The results depicted in Table 3.7 were obtained with an HPM preparation that was prepared as described in the materials and methods section. Less pure HPM, obtained by the method that was used for preparation of human breast tumor membrane preparations, yielded less stable membrane fractions unsuitable for quality control purposes. The isolation procedure probably did not purify adequately the membranes from endogenous proteases.
In the pilot quality control trials 10 laboratories received twice a set of 4 lyophilized samples in which the EGFR concentration was assayed using the HAP assay. ^{125}I-EGF was also lyophilized and dispatched. Results of this between-laboratory quality control are collected in Tables 3.8 and 3.9. The coefficient of variation ranged from 22-44%.

***Table 3.7** Effect of storage of lyophilized HPM on EGFR*

	Temperature of storage	Duration of storage (days)	EGFR (fmol/ml)
Sample A	4 °C	1	391
		35	447
		56	465
		84	403
		112	384
Sample B	4 °C	18	90 ± 7
	20 °C	18	80 ± 10

HPM preparations (lyophilized and not lyophilized) were stored at 4 °C and at 20 °C for various periods of time and assayed for EGFR content.

***Table 3.8** Four HPM samples (A-D) measured in 10 different laboratories (trial I)*

Lab.	A	B	C	D
1	108	41	120	43
2	87	80	89	70
3	93	40	90	44
4	66	37	72	41
5	86	43	100	43
6	59	69	152	76
7	54	52	92	25
8	75	26	73	28
9	51	20	65	25
10	61	69	72	50
Mean	74	48	93	45
S.D.	19	20	26	17
C.V. (%)	26	41	29	39

EGFR expressed in fmol/ml; A=C, B=D, A=2B

Table 3.9 Four HPM samples (A-D) measured in 9 different laboratories (trial II)

Lab.	A	B	C	D
1	63	50	26	16
2	35	41	13	12
3	43	51	22	12
4	68	62	27	10
5	58	66	20	25
6	41	32	15	10
7	46	39	23	26
8	56	37	16	13
9	45	48	14	23
Mean	51	47	20	16
S.D.	11	11	5	7
C.V.(%)	22	24	25	44

EGFR expressed in fmol/ml, A=B, C=D, A=2C

3.4 Discussion

Assaying EGFR in HPM, the HAP assay showed a good performance with a within-assay variation of 3.2-14% (Tables 3.1 and 3 5) and a between-assay variation of 8.6-13 5% (Table 3.1). The lower limit of detection was about 3 fmol/ml.
The impact of endogenously bound ligands on the outcome of the assay was suggested by Falette *et al.* (Falette *et al.* 1992) and therefore it was investigated whether the addition of hEGF to a HPM preparation affected the number of binding sites and the apparent Kd The results obtained indicate a reversible receptor binding interaction of hEGF. The effect of TGFα on the apparent Kd was somewhat less as compared to EGF. In the same experiment the occupation of EGFR by endogenous ligands was mimicked employing a centrifugation step. In all membrane preparations analyzed similar receptor values and nearly identical Kd's were observed These results demonstrated that the centrifugation step resulted in an almost complete elimination of the ligands. Those ligands occupying the receptors were still present in the centrifuged membrane preparation, but obviously did not affect measurable EGFR levels. The effect of the added ligands was in agreement with the competitive inhibition model Furthermore, acid treatment of tumor membranes did not increase measurable EGFR levels (Fig. 3 2a), as was claimed by Falette *et al.* (1992), who concluded that local production of EGF-like factors in breast tumors results in a masking of binding sites. However, Falette *et al.* expressed their results in fmol/mg of membrane protein. From our investigation it became clear that such an increase resulted from a selective

elimination of contaminating protein This could be concluded from the experiments in which both the acid treatment and membrane washing (neutral conditions) resulted in a significant decrease of the protein concentration in the membrane preparations, as a result of which the EGFR levels, if expressed as fmol/mg of membrane protein, increased The acid treatment therefore results merely in an enriched membrane preparation rather than in a detectable dissociation of endogenously bound ligands The question remains as to whether significant amounts of occupied receptors are present in a tumor membrane preparation While processing tumor tissue, a relatively large amount of buffer is normally used for homogenization purposes, thereby dissociating already a substantial amount of eventually present ligands from the receptors Moreover, in vivo the EGF receptor internalizes and degrades after occupation with ligand (downregulation) (Carpenter and Cohen 1990) If still small amounts of ligands are present in the membrane samples (in tumors containing very high levels of ligands), they do not interfere in a multiple point assay In a single point assay, however, the number of receptors will be underestimated due to the dilution of the specific radioactivity of the ^{125}I-EGF used by endogenous ligands

The data presented demonstrate that EGFR remains stable for at least 3 yr in cell membrane preparations stored at -80 °C, a finding which legitimizes the use of archived membrane preparations for the purpose of retrospective clinical studies on EGFR The stability of EGFR was also demonstrated in the experiments performed in order to obtain reference material for quality control purposes After lyophilization of membrane samples, which by itself resulted in a decrease of measurable specific binding sites in case lyophilization was performed in the absence of lyoprotectants, EGFR was stable for at least 16 weeks at 4 °C or for three weeks at ambient temperature.

Measurement of EGFR by means of a ligand binding assay was found to easily result in a false-negative outcome in cases where the membrane protein level falls below a certain threshold Using the HAP assay, this threshold was 0 2 mg/ml Using other procedures to separate bound and free ligand such as centrifugation or filtration, this threshold may be higher or lower than in the HAP assay Nevertheless, the HAP assay showed a much better overall performance than the generally used centrifugation and filtration assays

In the centrifugation assay, the higher apparent Kd's obtained suggest a more extended dissociation of EGF from the receptor as compared to the other two assays examined The dissociation in the centrifugation assay was initiated by the addition of 1 ml of assay buffer prior to the centrifugation step However, if the addition of buffer prior to the centrifugation step is omitted, this will lead to contamination of the bound fraction by free ligand, affecting the binding data (Munson 1983) The observation that similar receptor concentrations could be obtained in the tumor membranes using the centrifugation assay compared to the HAP assay, if both assays are performed using a multiple point titration procedure, indicates that quantitative pelleting of the membranes can be achieved with an ultracentrifugation (100,000 x g) step However, most investigators who perform centrifugation for separation of bound and free ligand, use much lower centrifugation forces, as was shown in Table 2 1 In those cases quantitative precipitation of all membranes present is not achieved (Dittadi *et al.* 1990) yielding underestimated EGFR levels

Membrane preparations containing relatively high levels of EGFR could be analyzed properly using the filtration assay Apparent Kd's were similar as compared to the HAP

assay, since in both assays rapid washing steps could be performed. However, using the filtration assay for membranes containing the lower EGFR levels, such as in the serial diluted HPM samples, no reliable filtration assays could be performed due to the huge scattering of the data points. As a consequence, false negative assay results easily occur by employing the filtration assay. Furthermore, the filtration assay was very time-consuming and is therefore unsuitable to perform large series of samples simultaneously. On the other hand, the HAP assay was the easiest to use in performing large series of measurements simultaneously. The use of HAP improves the performance of an EGFR radioligand binding assay.
A relatively large variation was observed in the between-laboratory quality control trials as compared to the small variation observed in the within-assay examinations. The use of an internal standard preparation indicates that this variation can be improved significantly. In order to achieve more uniformity between laboratories, an effort to continue the between-laboratory quality control trials is undertaken. The standardization of EGFR assays is important for comparison of EGFR data, a prerequisite when trying to determine the significance of EGFR as a prognostic marker in human breast cancer and its role in tumor biology. The data presented in this chapter suggest that the HAP assay is acceptable as a standard technique for measurement of EGFR.

References are listed on pages 99-110.

CHAPTER 4

RADIOIODINATION OF EPIDERMAL GROWTH FACTOR

Radioiodinated epidermal growth factor (EGF) is generally used in EGF Receptor (EGFR)-binding studies (e.g. Carpenter *et al.* 1975, Hock and Hollenberg 1980, Magun *et al.* 1982, Pérez *et al.* 1984, Fitzpatrick *et al.* 1984, Lin *et al.* 1987, Nicholson *et al.* 1988, Marti *et al.* 1989). EGF contains five tyrosine residues (Savage *et al.* 1972) that allow formation of various labeled EGF species during iodination. Indeed, reversed-phase HPLC studies by Matrisian *et al.* (1985) demonstrated that iodination of homogeneous EGF by Chloramine T results in a heterogeneous ^{125}I-EGF preparation. Moreover, some tyrosine residues in the EGF molecule may be involved in receptor binding (Engler *et al.* 1990, 1991, Campion *et al.* 1990), which implies that iodination might profoundly affect ligand-receptor interaction. Investigators rarely test whether the radioiodinated EGF preparation behaves the same as the natural ligand in receptor binding (Kermode 1988), although receptor affinity and capacity estimates can be seriously affected when there is no equivalence between the labeled and the native ligand (Kermode 1988, Taylor 1975, Hollemans and Bertina 1975). Therefore, if the receptor-binding data are to yield realistic information on the nature of the ligand-receptor interaction, identical receptor-binding affinity of labeled and unlabeled EGF is necessary. This is particularly true for studies in which receptor-binding data are obtained by competition between labeled and unlabeled EGF (Kermode 1988).

Various methods have been described for preparing radioiodinated EGF. Iodination of EGF has been established either directly, by oxidation of the radioiodide in the presence of the polypeptide (Carpenter *et al.* 1975, Hock and Hollenberg 1980, Korc and Finman 1989, Marti *et al.* 1989), or indirectly, for human EGF, by conjugating EGF with a radioiodine-containing acylating agent (Bolton-Hunter reagent) (Bolton and Hunter 1973, Kermode and Tritton 1990). The oxidation of the radioiodide can be performed either chemically or enzymatically. For chemical oxidation, various agents are available, of which Chloramine T has been used the most extensively (e.g. Hock and Hollenberg 1980, Magun *et al.* 1982, Fitzpatrick *et al.* 1984, Pekonen *et al.* 1988). Despite its widespread acceptance, however, the Chloramine T method, in which the oxidizing agent is in solution, appears to damage the polypeptide to be iodinated (Sherman *et al.* 1974, Bolton *et al.* 1979, Lee and Griffiths 1984, Bolton 1985). In contrast, methods involving a sparingly soluble oxidizing agent (Iodogen®) or agents coupled to a solid support (Iodo-beads®, Protag-125®) have been claimed to be more gentle because direct contact between the oxidizing agent and the protein to be radiolabeled is minimized (Fraker and Speck 1978, Slacinski et al 1980, Markwell 1982, Guenther and Ramsden 1984, Nicholson *et al.* 1985, Korc and Finman 1989). Enzymatic oxidation of the radioiodide with lactoperoxidase can also be achieved both in the solubilized state (Marchalonis 1969, Thorell and Johansson 1971) and, more mildly, in the solid state (David and Reisfeld 1974, Marti *et al.* 1989). In the latter case, Sepharose-coupled lactoperoxidase (29) or lactoperoxidase and glucose oxidase immobilized on hydrophilic spheres (Enzymobeads®) are used.

In the present study, we performed six different methods for direct iodination of mouse EGF. The binding behavior of the different radioligands to human placental membranes (HPM) containing EGFR was investigated by comparing the saturation binding curves (increasing concentrations of ^{125}I-EGF) and the competitive binding curves (competition of ^{125}I-EGF with unlabeled EGF) of the various products in an attempt to establish whether receptor-binding equivalence could be achieved between

radioiodinated EGF and its unlabeled counterpart. Also the binding behavior of different ^{125}I-EGF column fractions, obtained after preparative separation by HPLC of Chloramine T- and Enzymobeads-labeled EGF, was evaluated to investigate whether a homogeneous radioligand exhibiting ligand equivalence could be obtained by HPLC purification. The effect of nonequivalence in binding behavior between labeled and unlabeled EGF was investigated by performing Scatchard analysis of saturation and competitive binding data. We also assessed maximal binding capacity of each ^{125}I-EGF preparation to HPM, i.e., the fraction of the tracer that would bind at infinite receptor concentrations, to observe whether nonequivalence in binding behavior between labeled and unlabeled EGF is associated with a decrease in binding capacity of the ^{125}I-EGF preparation.

4.2 Materials and methods

4.2.1 Materials

Receptor grade mouse EGF was obtained from Bioproducts for Science, Inc. (Indianapolis, IN, U.S.A.). Carrier-free Na^{125}I and commercially prepared ^{125}I-labeled mouse EGF were obtained from Amersham International plc. (Amersham, Buckinghamshire, U.K.). Iodogen and Iodo-beads were purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.). Chloramine T, sodium disulfite and iodine monochloride (ICl) were obtained from Merck (Darmstadt, Germany). Enzymobeads and hydroxylapatite (HAP; DNA grade Biogel HTP) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). β-D-Glucose was supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bacitracin was obtained from Serva (Heidelberg, Germany). Bovine serum albumin was purchased from Behringwerke AG (Marburg/Lahn, Germany). Protag-125 and disposable 3-ml C_{18} solid-phase extraction (SPE) columns (200 mg of C_{18}) were obtained from J.T. Baker, Inc. (Phillipsburg, NJ, U.S.A.). Protag-125 is not available anymore.

4.2.2 Iodination methods

Iodinations were carried out at room temperature with ±10 µg of EGF in 10 µl of 50 mM phosphate buffer, pH 7.4, and with 10 µl (1 mCi) of Na^{125}I (EGF/^{125}I molar ratio = about 3). To remove unreacted iodine after the iodination reaction, the reaction mixture was loaded onto a C_{18} SPE column, pre-equilibrated sequentially with 4 ml each of methanol, water, and the respective dilution buffer (see the respective iodination reactions below). The SPE column was washed three times with 1 ml of an aqueous solution of 20% methanol and 0.1% trifluoroacetic acid (TFA). ^{125}I-EGF was eluted from the column with 1 ml of 80% methanol, 0.1% TFA. The efficiency of iodine incorporation was obtained by dividing the radioactivity eluted in the protein fraction by the total radioactivity added. The methanol was evaporated under a stream of dry nitrogen in a water bath at 37 °C. The ^{125}I-EGF solution resulting was brought to a final volume of 1 ml with EGFR assay buffer (20 mM phosphate buffer, pH 7.4, containing 150 mM NaCl, 50 µM bacitracin, and 0.1 mg/ml BSA). The recovery of EGF protein

was approximately 45%.
To minimize adsorption of ^{125}I-EGF to hydroxylapatite (HAP) and to minimize nonspecific binding, the ^{125}I-EGF preparations were pre-incubated with 200 µl of HAP suspension for 30 min at 20 °C. HAP was used to separate receptor-bound and free EGF in the binding experiments (see below). After centrifugation for 2 min at 800 x g (20 °C), the tracer preparation was pipetted away from the HAP pellet. The amount of adsorption of the pretreated ^{125}I-EGF preparations to HAP (Blank HAP binding) was determined by incubating about 250,000 cpm of tracer with 100 µl of HAP suspension and counting the radioactivity of the HAP-bound ^{125}I-EGF after two washes with 1 ml of EGFR assay buffer without BSA and subsequent centrifugation for 2 min at 800 x g and decanting of the supernate.
Approximately 5 x 10^6 cpm of the ^{125}I-EGF preparation was adjusted to a volume of 200 µl with water; then 50 µl of acetonitrile was added, and the ^{125}I-EGF preparation was subjected to HPLC analysis. For preparative separation of ^{125}I-EGF, we performed two iodination reactions simultaneously. The ^{125}I-EGF preparations obtained were then pooled before the HPLC step.

4.2.2.1 Chloramine T method

The Chloramine T method was performed by a modification of the method of Matrisian *et al.* (1985). To 10 µl of EGF, 170 µl of 50 mM phosphate buffer (pH 7.4) and 10 µl (1 mCi) of Na^{125}I were added. The reaction was started by the addition of 20 µl of Chloramine T (2 mg/ml). After 25 s of incubation 20 µl of sodium disulfite (4 mg/ml) was added to stop the reaction. Then 1 ml of 50 mM phosphate buffer (pH 7.4) was added to the reaction tube and unincorporated iodine was immediately removed by using SPE columns as described above. The efficiency of iodine incorporation was 45-55%, which corresponds to about 0.3-0.4 iodine atomes incorporated per molecule of EGF.

4.2.2.2 Iodogen method

According to the manufacturer's recommended procedure, to 10 µl of EGF, 20 µl of 100 mM borate buffer (pH 8.0) and 10 µl (1 mCi) of Na^{125}I were added. The reaction was started by pipetting the mixture into a 10 x 75 mm borosilicate glass tube coated with 1 µg of Iodogen. After 10 min of incubation, 1 ml of 100 mM borate buffer (pH 8.0) was added, after which the mixture was pipetted out of the Iodogen-coated tube to stop the reaction. Unreacted iodine was removed as described above. Radioiodine was incorporated with 45-50% efficiency.

4.2.2.3 Iodo-beads method

According to the manufacturer's instructions, we mixed 10 µl of EGF with 180 µl of 100 mM phosphate buffer (pH 7.0) and 10 µl (1 mCi) of Na^{125}I. The reaction was initiated by the addition of one Iodo-bead. After two min of incubation, 1 ml of 100 mM phosphate buffer (pH 7.0) was added and the reaction was stopped by loading the

reaction mixture without the Iodo-bead onto a C_{18} SPE column. Free iodine was removed as described above. The efficiency of the Iodo-bead iodination reaction was 40-55%.

4.2.2.4 Iodine monochloride method

The iodine monochloride method was performed as described by Contreras *et al.* (1983). The molar ratio of ICl to EGF was 2:1: approximately 3.4 nmol of ICl in 20 µl of 0.85 % NaCl solution was added to a mixture of 10 µl of EGF (10 µg = 1.7 nmol), 100 µl of 200 mM borate buffer containing 160 mM of NaCl (pH 8.0), and 20 µl (2 mCi) of $Na^{125}I$, while vortex-mixing. After vortex-mixing for 1 minute adding 1 ml of 200 mM borate buffer and 160 mM NaCl (pH 8.0), the mixture was loaded onto a C_{18} SPE column as described above to remove unincorporated iodine. The fraction of iodine incorporated into EGF ranged from 20% to 30%.

4.2.2.5 Protag-125 method

Iodination of EGF by using Protag-125, an insoluble glycouril agent (Guenther and Ramsden 1984), was performed by adding a mixture of 10 µl of EGF solution, 20 µl of 100 mM borate buffer (pH 8.4) and 10 µl (1 mCi) of $Na^{125}I$ into a borosilicate glass tube containing 1.7 mg of Protag-125. After 6 min of incubation with intermittent mixing of the suspension for 5 s at 30-s intervals, 1 ml of 50 mM phosphate buffer (pH 7.4) was added. The Protag beads were sedimented at 1 x g, after which the supernate was loaded onto an SPE column for removal of free iodine as described above. The efficiency of the Protag iodination reaction was 30-40%.

4.2.2.6 Lactoperoxidase-glucose oxidase method (Enzymobeads)

Iodination was performed according to the manufacturer's instructions. 10 µl of EGF, 50 µl of 200 mM phosphate buffer (pH 7.4), 50 µl of Enzymobead reagent, and 10 µl (1 mCi) of $Na^{125}I$ were mixed and 25 µl of 1% β-D-glucose was added to start the reaction. After incubation for 10 min, 1 ml of 200 mM phosphate buffer (pH 7.4) was added. Unbound iodine was removed by SPE as described above. Iodine was be incorporated with 40-50% efficiency.

4.2.3 Specific radioactivity and ligand equivalence

The specific radioactivity of ^{125}I-EGF preparations was determined by the "self-displacement" method (Morris 1976, Calvo *et al.* 1983), plotting Bound/Total (B/T) ratios versus radioactivity ^{125}I-EGF or mass of unlabeled EGF added to the receptor preparation. Separation of free and receptor-bound ligand was performed by using HAP. HAP adsorps EGFR protein, leaving nonbound EGF in solution. Only low-speed centrifugation is required subsequently for the separation step.
Two sets of binding experiments, using EGFR-containing HPM prepared as described

in chapter 2, were performed. In one set, providing the saturation curve, 100 μl of HPM (30 μg of membrane protein) was equilibrated in duplicate incubations with increasing concentrations of ^{125}I-EGF (ranging from 2 x 10^4 to 3 x 10^6 cpm) in a final volume of 140 μl of EGFR assay buffer at 20 °C for 16-20 h. In the second set of binding experiments, which provided the competitive binding curve, 100 μl of HPM was equilibrated in duplicate incubations with a fixed concentration of labeled EGF (about 2 x 10^4 cpm) and increasing concentrations of unlabeled EGF (from 0 to 3500 fmol/tube) at 20 °C for 16-20 h. To separate free and receptor-bound ligand, 100 μl of HAP suspension was added to each assay tube. The tubes were equilibrated for another hour at 20 °C with intermittent mixing to ensure new equilibrium. After incubation and centrifugation for 2 min at 800 x g (20 °C), the supernate was decanted. The HAP pellets were washed twice with 1 ml of EGFR assay buffer without BSA and centrifuged for 2 min at 800 x g (20 °C). The HAP pellets were counted for radioactivity in a Model 1261 multi gamma counter (LKB-Wallac, Turku, Finland) with 82.5% efficiency.

According to Van Zoelen (1992), in a system in which a homogeneous set of binding sites is equilibrated with either labeled ligand (L^*) of unlabeled ligand (L^u), which may differ in their receptor-binding affinity, the ratio of the corresponding dissociation constants K^* and K^u is related to the added concentrations of L^* and L^u, which lower the initial binding to the same B/T ratio, according to:

$$\frac{L^u - L^*}{L^*(1-B^*/T)} = \frac{K^u - K^*}{K^*}$$

In this equation B^* is the experimentally obtained value for bound labeled ligand under such equivalent competition conditions, while in addition it is assumed that nonspecific binding components are negligible. Since L^* is only known in cpm-values (C^*), which are related by the specific radioactivity of the labeled ligand preparation ($1/\delta$), it follows that

$$\frac{L^u}{C^*} = \delta \frac{K^u}{K^*} + \frac{\delta B^*}{T} (1-K^u/K^*)$$

Thus, by plotting L^u/C^* (fmol/cpm) for various values of B^*/T, a linear plot is derived from which the values of both δ and K^u/K^* can be obtained from the intercept with ordinate at B^*/T-values 0 and 1. This equation is analogous to the one derived for a homogeneous set of binding sites (Hollemans and Touber 1974, Hollemans and Bertina 1975).

Therefore, in this study the binding data of the saturation curve were transformed into B/T ratios and plotted on a semilogarithmic scale against the radioactivity added to the receptor preparation, after subtraction of the initial 2 x 10^4 cpm added in the first tube. For the competitive binding curve, the binding data were transformed into B/T ratios and plotted against femtomoles of unlabeled EGF added, in the same semilogarithmic plot. At several B/T ratios in this plot, cpm of ^{125}I-EGF and femtomoles of unlabeled EGF were read from the saturation curve and the competition curve, respectively.

Then femtomoles obtained at several B/T ratios were divided by the cpm of labeled EGF which lowered the B/T to the same extent. Plotting the ratio of fmol/cpm at several B/T ratios vs the corresponding B/T ratio reveals the Ligand Equivalence (LE) plot. For a radioligand which has the same affinity as compared to that of the unlabeled ligand ($K^* = K^u$), the obtained line in the LE plot is horizontal (Hollemans and Touber 1974, Hollemans and Bertina 1975, Van Zoelen 1992). There is nonequivalence in binding behavior between labeled and unlabeled ligand when the slope of this line differs significantly from zero. The specific radioactivity of the tracer preparation can then be obtained by extrapolation of the straight line to B/T=1, whereas the ratio of the intercepts at B/T=1 and B/T=0 represents the ratio of the Kd's of unlabeled and labeled ligand.
In the case that multiple receptor classes are present, the LE plot is horizontal if there is equivalence in binding behavior between labeled and unlabeled ligand, i.e. that the dissociation constants of the labeled and unlabeled ligand for both high- (K^*h and K^uh) and low-affinity receptors (K^*l and K^ul) are identical. In the case in which there is nonequivalence but the ratios of the dissociation constants of the labeled and unlabeled ligand are similar for both the high- and low-affinity receptor class ($K^*h/K^uh = K^*l/K^ul$) , a nonhorizontal but straight line is obtained. In cases in which K^*h/K^uh not equals K^*l/K^ul, a complicated sigmoidal LE plot is obtained (Van Zoelen 1992).
Another plot can be constructed from the obtained binding data. When cpm of ^{125}I-EGF added, read from the saturation curve at several B/T ratios, are plotted versus fmoles of unlabeled EGF, read from the competitive binding curve at corresponding B/T ratios, a straight line is obtained when there is equivalence between labeled and unlabeled ligand, in which the slope represents the specific radioactivity of the tracer preparation in cpm/fmol (Calvo *et al.* 1983)

4.2.4 Reversed-phase HPLC

The HPLC apparatus included an SP8700 solvent delivery system and an SP8750 organizer (Spectra-Physics, Santa Clara, CA), a Model 470 scanning fluorescence detector (Millipore, Waters Associates, Bedford, MA) and a Model 201-202 fraction collector (Gilson, Villiers-le-Bel, France). ^{125}I-EGF preparations were chromatographed using a 0.39 x 30 cm μBondapak C_{18} reversed-phase column (Millipore, Waters) connected to a μBondapak C_{18} guard column, with a linear gradient changing from 20% to 31.7% acetonitrile (balance 0.1% trifluoroacetic acid) over 70 minutes at 20 °C. The solvent flow was maintained at 2 ml/min. Fractions (1 ml) were collected and counted for radioactivity. In those experiments where nonradioactive EGF was chromatographed, we used the fluorescence of endogenous tryptophan (excitation wavelength 215 nm, emission wavelength 340 nm) for detection.

4.2.5 Maximal binding capacity

About 2 x 10^4 cpm of EGF was equilibrated in duplicate with increasing amounts of HPM (4-100 μg of membrane protein), containing about 6000 fmol of EGFR/mg of protein, in a total volume of 140 μl of EGFR assay buffer at 20 °C for 16-20 h. Separation of receptor-bound and free was performed under nondissociating

conditions: 100 µl of the incubate was added to an HAP pellet, which was obtained by centrifuging 100 µl of HAP suspension and subsequently aspirating the supernate. After incubation for 1 h at 20 °C with repeatedly mixing, the samples were centrifuged for 2 min at 800 x g (20 °C). Then 50 µl of the supernate was counted for radioactivity (representing 50% of the free ligand) and the bound fraction was calculated by subtracting the nonbound ^{125}I-EGF from the total radioactivity added to the HAP pellet. A plot of 1/radioactivity bound vs 1/volume of the membrane preparation was constructed, such that the ordinate intercept represented the fraction of ^{125}I-EGF that would bind at infinite receptor concentrations (maximal binding capacity) (Kermode and Tritton 1988, Calvo *et al.* 1983).

4.3 Results

4.3.1 Specific radioactivity and ligand equivalence

Mouse EGF was iodinated with Na^{125}I by using six direct radioiodination methods. The binding behavior of the radioiodinated EGF preparations was assessed by binding studies with EGFR-containing HPM.

For EGF labeled with Chloramine T, the competitive binding curve, in which unlabeled EGF increased, shows a steeper decline in binding than does the saturation curve, in which only labeled ligand was added (Fig. 4.1a). From the saturation curve in Fig. 4.1a, cpm of ^{125}I-EGF added were read at various B/T ratios. From the competitive binding curve, femtomoles of unlabeled EGF added were read at the same B/T ratios. The cpm of ^{125}I-EGF obtained were plotted vs the corresponding fmoles of unlabeled EGF. This plot shows upward curvilinearity (Fig. 4.1b), which precludes a reliable estimation of the specific radioactivity. From the data shown in Fig. 4.1a, another plot was constructed (Fig. 4.1c) in which the ratio fmol/cpm at several B/T ratios was plotted vs the corresponding B/T ratio, providing the ligand equivalence (LE) plot. As shown in Fig. 4.1c, the slope of this line differs significantly from zero, which implies that the affinity of the Chloramine T labeled ^{125}I-EGF was lower as compared to that of the unlabeled EGF. Attempts to determine the specific radioactivity of Chloramine T-^{125}I-EGF by extrapolation to B/T=1 failed, since these plots showed curvilinearity.

Using commercially prepared ^{125}I-EGF (Chloramine T method), the results of these binding experiments were essentially identical to those shown in Fig. 4.1 obtained with Chloramine T-^{125}I-EGF, prepared in our laboratory.

In the literature iodinated ligands prepared with Iodogen or Iodo-beads have been described as undergoing a milder iodination procedure (Fraker and Speck 1978, Markwell 1982). In the present study it appeared that binding experiments performed with ^{125}I-EGF labeled with Iodogen (n=5) or Iodo-beads (n=4) yielded essentially identical binding curves as were obtained with Chloramine T ^{125}I-EGF (Fig. 4.1a), i.e. diverging saturation and competition curves and nonhorizontal LE plots (data not shown). On the other hand, parallel binding curves were obtained when we used ^{125}I-EGF labeled by the ICl method (Fig. 4.2a). As a consequence of this parallelism, a perfectly straight line was obtained with cpm of ^{125}I-EGF plotted vs femtomoles of unlabeled EGF (Fig. 4.2b), and a straight and horizontal LE plot could be constructed

(Fig. 4.2c), indicating equivalence in binding behavior between labeled and unlabeled EGF. The specific radioactivity of ICI-^{125}I-EGF could be calculated accurately from the slope of the line in Fig. 4.2b. The specific radioactivity of ICI-^{125}I-EGF preparations was 415 ± 123 Ci/mmol (mean ± SD, n=5)

Equivalence in binding behavior between labeled and unlabeled EGF could also be observed by using ^{125}I-EGF, iodinated with Protag-125 and Enzymobeads. The specific radioactivities were 525 ± 146 Ci/mmol (n=9) and 570 ± 80 Ci/mmol (n=7) for ^{125}I-EGF labeled with Protag and Enzymobeads respectively.

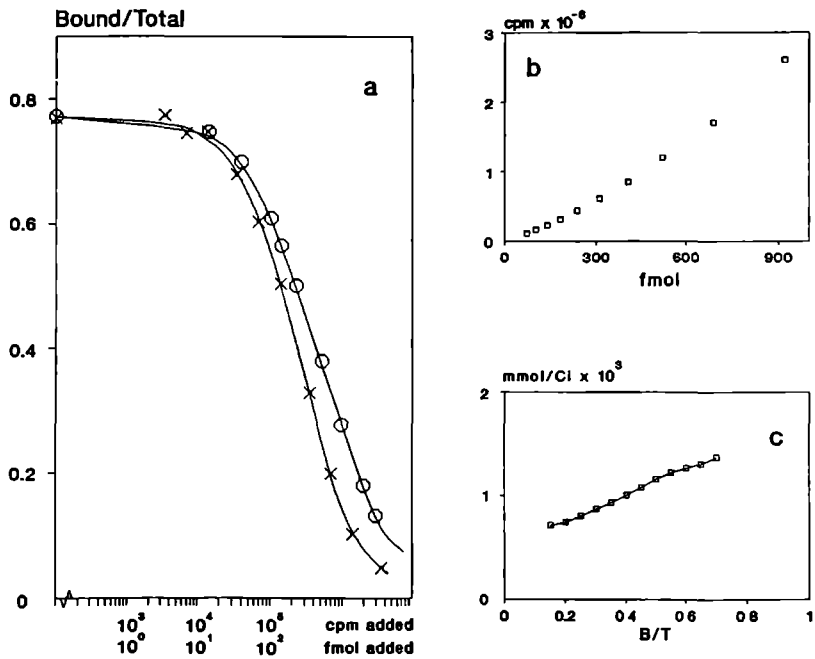

Fig. 4.1. Binding experiments of a Chloramine T-^{125}I-EGF preparation with an HPM preparation a, Binding curves obtained from experiments performed as described in materials and methods (o, cpm of ^{125}I-EGF added, x, femtomoles of unlabeled EGF added), b, radioactivity added was plotted against the corresponding mass of unlabeled EGF at several points where the B/T ratio was the same for both curves in Fig 4 1a, c, LE plot obtained by plotting the ratio fmol/cpm at various B/T ratios against the corresponding B/T ratio

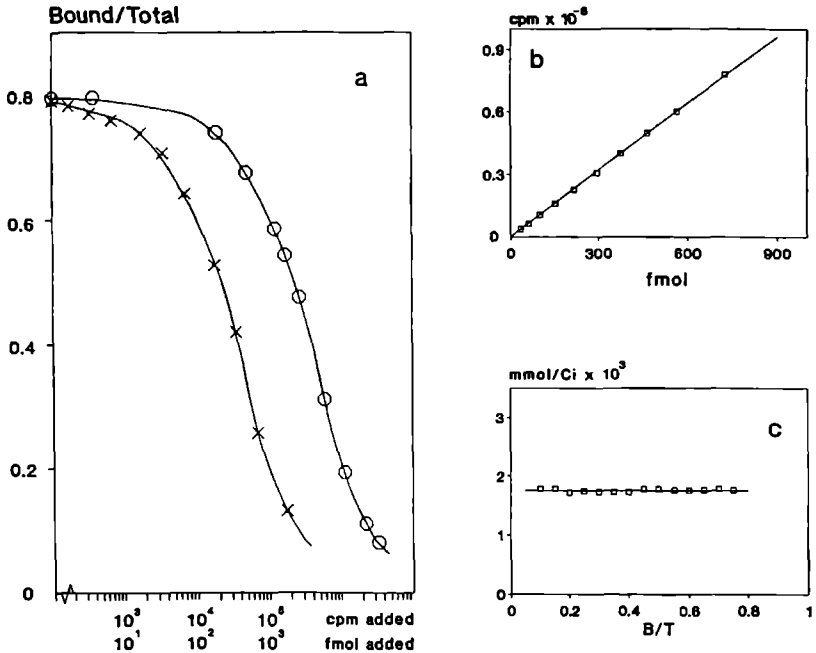

Fig. 4.2 *Determination of the specific radioactivity and relative affinity of ICI-^{125}I-EGF.*
o, increasing tracer concentrations; x, increasing concentrations of cold EGF. For a to c, see legend Fig. 4.1.

4.3.2 Reversed-phase HPLC

As shown in the HPLC elution profiles (Fig. 4.3), all six iodination methods used resulted in a heterogeneous ^{125}I-EGF preparation. However, EGF that was iodinated using Chloramine T, Iodogen, or Iodo-beads (Fig. 4.3a-c) showed the predominant peaks at shorter retention times on HPLC than unlabeled EGF. For EGF labeled with use of Enzymobeads, Protag-125, or ICI, the predominant peaks were shifted to longer retention times (Fig. 4.3d-f). The HPLC elution profile of commercially available

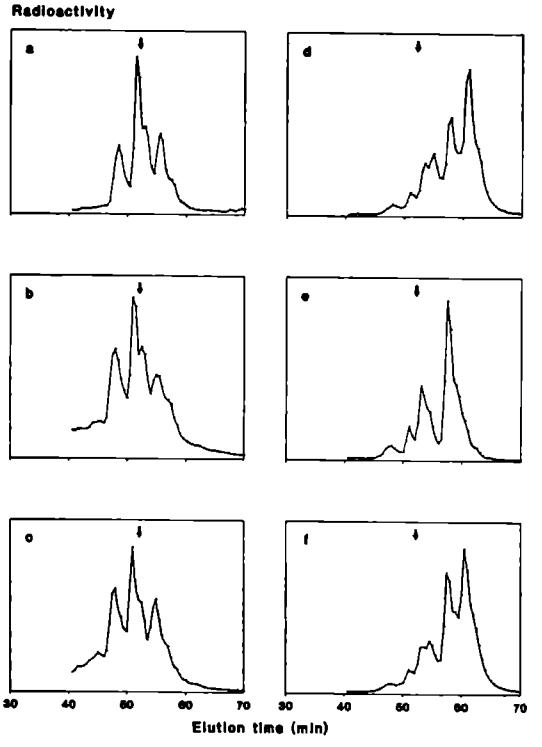

Fig. 4.3. C_{18} reversed-phase HPLC elution profiles of ^{125}I-EGF, prepared with six different radioiodination methods Approximately 5 x 10^6 cpm ^{125}I-EGF were subjected to HPLC Fractions of 1 ml were collected and counted for radioactivity a, Chloramine T, b, Iodogen, c, Iodo-beads, d, Enzymobeads, e, Protag-125, f, Iodine monochloride Unlabeled EGF eluted as indicated by arrows

radioiodinated mouse EGF (Chloramine T method) was essentially identical to that shown in Fig 4 3a Neither the use of smaller amounts of Chloramine T, Iodogen, or Iodo-beads, nor iodination for shorter incubation times changed the HPLC profiles for iodinated EGF significantly (data not shown)

To study the effect of the nonradioactive reagents per se on the chromatographic properties of EGF, we performed the reactions under the conditions described for iodination, but with no radioiodide (Fig 4 4) The HPLC apparatus in this case was

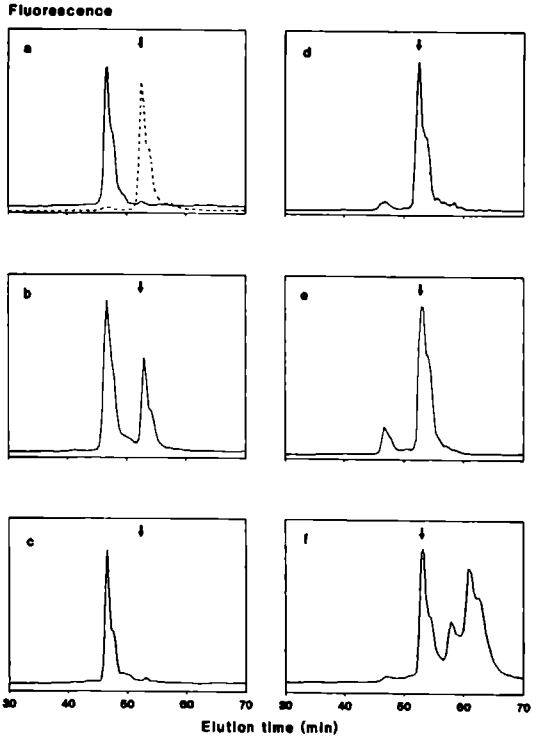

Fig. 4.4. C_{18} reversed-phase HPLC elution profiles of EGF, treated with the various radioiodination reagents, in the absence of radioiodide. 10 µg EGF was treated and injected onto the HPLC column. EGF protein in the eluent was monitored by endogenous tryptophan fluorescence. Unlabeled EGF eluted as indicated by the dashed line and by arrows. a-f as in Fig. 4.3.

equipped with a fluorescence detector. Treating EGF with the Chloramine T, Iodogen, or Iodo-beads reagents resulted in shorter retention times (Fig. 4.4a-c). In contrast, treating EGF with the Enzymobeads or Protag-125 reagents (Fig. 4.4d, e) did not affect the elution position of the protein, except for a slight increase of the first peak in the elution profile of Protag-treated EGF. Treatment of EGF with nonradioactive ICl yielded several fluorescent peaks, one with the retention time of native EGF and others with longer retention times (Fig. 4.4f), probably from the incorporation of nonradioactive iodine from the ICl molecule into the EGF.

Oxidation of EGF with hydrogen peroxide also produced a shift to shorter retention times, comparable to the treatment of EGF with Chloramine T, Iodogen, and Iodobeads Moreover, oxidation of ICl-^{125}I-EGF by hydrogen peroxide, also shifted the various peaks to shorter retention times (Fig. 4 5)

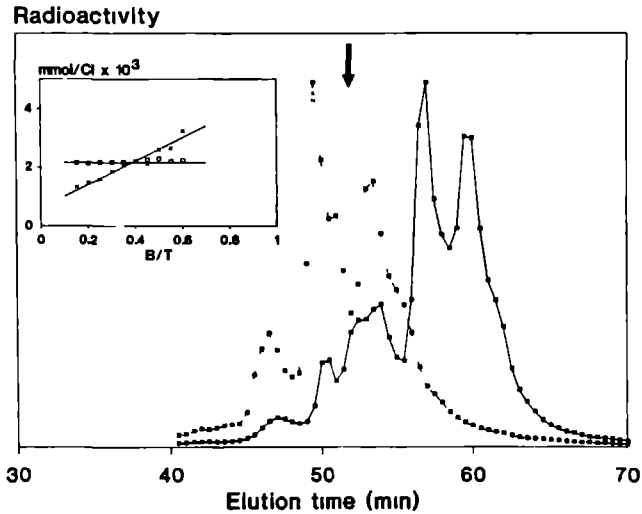

Fig. 4.5. Effect of oxidation of ICl-^{125}I-EGF by hydrogen peroxide
(-) HPLC profile of the nonoxidized ICl-^{125}I-EGF We also added 30 µl of a 30% H_2O_2 solution to a$_{1/2}$ ox. 2 5 µg of ^{125}I-EGF in 1 ml of assay buffer After 30 min incubation at room temperature, 5 x 10^6 cpm of the oxidized ^{125}I-EGF were subjected to HPLC (–) Insert LE plots of the nonoxidized (□) and the H_2O_2 oxidized (x) ^{125}I EGF

4.3.3 Ligand equivalence analysis (LEA) of different HPLC column fractions

To investigate the binding behavior of ^{125}I-EGF entities eluting with different retention times on HPLC, Chloramine T- and Enzymobeads-^{125}I-EGF were chromatographed preparatively. HPLC column fractions (1 ml) were collected and counted for radioactivity. Four peaks (Fig. 4 6) were analyzed separately Each peak was rechromatographed on HPLC (see inserts of Fig. 4 7 and 4 8) and analyzed by LEA, as described in Materials and Methods As the sloping lines in Fig 4 7 show, all four ^{125}I-EGF fractions obtained after chromatography of Chloramine T-^{125}I-EGF had lower affinity for the placental EGF receptor than unlabeled EGF.
The binding behavior of the pooled Enzymobeads-^{125}I-EGF preparations was also investigated by LEA. Three fractions (E2, E3 and E4) obtained after HPLC contained

^{125}I-EGF that was equivalent to the native EGF in receptor binding, as can be concluded from the horizontal LE plots (Fig 4 8b-d), therefore, these fractions could be used for accurate determinations of specific radioactivity In contrast, LEA of the E1 tracer resulted in a nonhorizontal plot, indicating nonequivalence (Fig 4 8a) This E1 tracer could have been generated by iodination of already oxidized EGF entities present in the unlabeled EGF preparation (Fig 4 4a, dashed line) The overall nonchromatographed ^{125}I-EGF preparation (E0) showed ligand equivalence, from which it appears that no interference is encountered from the presence of the small amount of the nonequivalent E1 tracer Another mild radioiodination procedure such as ICl, also yielded a tracer with overall ligand equivalence (Fig 4 5, insert) Oxidation of this ICl-^{125}I-EGF preparation by hydrogen peroxide introduced nonequivalence in binding behavior between labeled and unlabeled EGF (Fig. 4.5, insert)

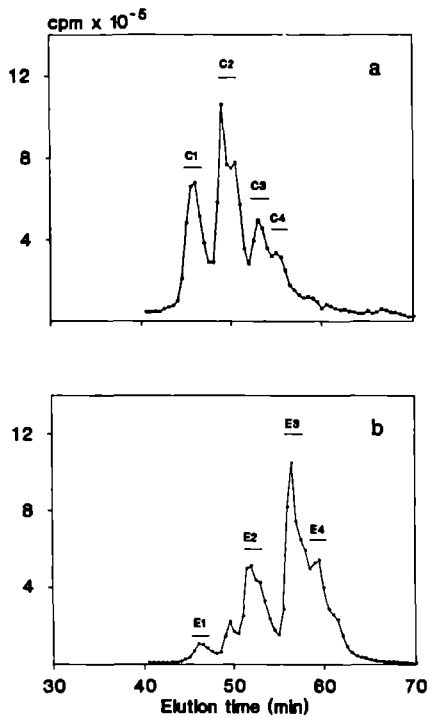

Fig. 4.6. Preparative isolation HPLC elution profiles of Chloramine T-^{125}I-EGF (a) and Enzymobeads-^{125}I-EGF (b) Column fractions were pooled as indicated by C1-C4 and E1-E4

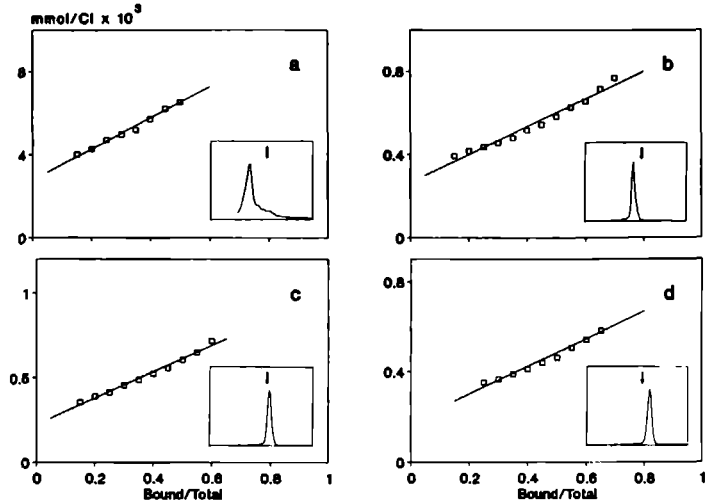

Fig. 4.7. Ligand equivalence plots of four isolated Chloramine T-^{125}I-EGF preparations obtained by preparative separation by HPLC. a, C1; b, C2; c, C3; d, C4. (see Fig. 4.6).
The HPLC profiles of the distinct ^{125}I-EGF preparations are shown in the inserts.

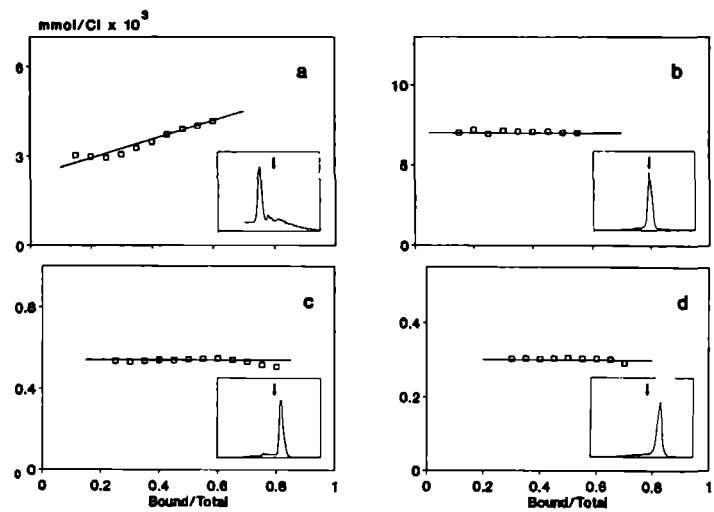

Fig. 4.8. LE plots of four isolated Enzymobeads-^{125}I-EGF preparations obtained by preparative separation by HPLC. a, E1; b, E2; c, E3; d, E4. (see Fig. 4.6)
The HPLC profiles of the distinct ^{125}I-EGF preparations are shown in the inserts.

The specific radioactivities of the equivalent fractions E2, E3, and E4 varied widely, being 144, 1877, and 3296 Ci/mmol respectively. Fully in agreement with this observation was the finding by HPLC analysis with fluorescence detection of "cold iodinated" EGF (iodination with nonradioactive iodine) that nearly all fluorescence intensity was located in the E2 area (data not shown).

4.3.4 Scatchard analysis

Because the specific radioactivity of commercially supplied Chloramine T-^{125}I-EGF preparations is given by the manufacturer (established by other procedures), we could perform Scatchard analyses (Scatchard 1949) of saturation and competitive binding data to observe the effect of using labeled or unlabeled EGF as a variable in an EGFR assay As demonstrated in Fig. 4 9a, the apparent number of binding sites obtained from the competitive analysis was lower than the apparent number of binding sites obtained when we used increasing concentrations of ^{125}I-EGF (24 fmol/ml and 49 fmol/ml respectively). Moreover, the apparent dissociation constant (Kd) was considerably higher when we used the latter method (0 20 nM and 0 65 nM respectively).

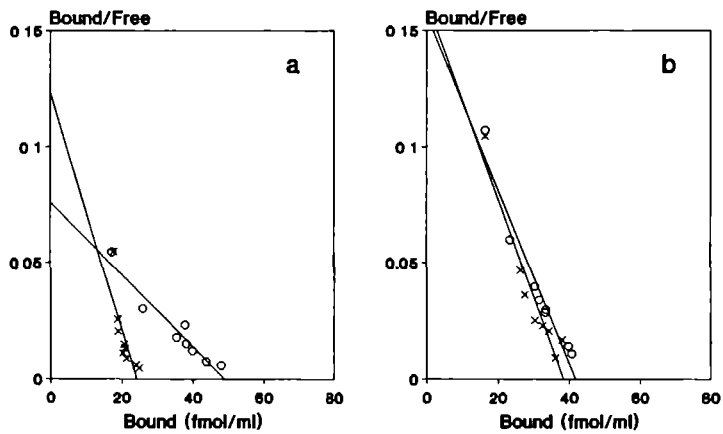

Fig. 4.9. *a, Scatchard plots obtained from binding studies, with increasing concentrations of commercially obtained ^{125}I-EGF (o) and increasing concentrations of unlabeled EGF (x), b, Scatchard plots obtained from saturation (o) and competition (x) binding experiments by using ICI-^{125}I-EGF 2 5 µg HPM protein was equilibrated with EGF, 0 15-3 5 nM, final concentration in a total volume of 140 µl of EGFR assay buffer Specific binding (Bs) was calculated as the difference between total binding and nonspecific binding, assessed by parallel incubations in the presence of 250-fold excess of unlabeled EGF B/F = specifically bound/free ratio*

Table 4.1. *Scatchard analysis (saturation and competitive binding data) for nonchromatographed Enzymobeads-^{125}I-EGF (E0) and the HPLC column fractions of Enzymobeads-^{125}I-EGF (E2, E3, and E4).*

	Saturation analysis		Competition analysis	
	N (pM)	Kd (nM)	N (pM)	Kd (nM)
E0	927	0.21	887	0.20
E2	973	0.11	1085	0.13
E3	1028	0.10	1050	0.11
E4	898	0.15	982	0.18

See legend to Fig. 4.9. N = number of receptors; Kd = dissociation constant.

Scatchard plots derived from saturation and competitive binding data obtained with ^{125}I-EGF labeled by the ICI method did not show significant differences with respect to the apparent number of receptors (42 fmol/ml and 39 fmol/ml respectively) and apparent dissociation constants (0.27 nM and 0.24 nM respectively) (Fig. 4.9b). Similar to observations for ICI-^{125}I-EGF, Scatchard plots obtained from saturation and competitive binding data with ^{125}I-EGF labeled with Protag or Enzymobeads were not different (data not shown).
Furthermore, saturation and competitive binding experiments using nonchromatographed Enzymobeads-^{125}I-EGF (E0) or the HPLC purified ^{125}I-EGF preparations E2, E3 and E4 resulted in similar number of receptors (N) and apparent Kd by Scatchard analysis (Table 4.1).

4.3.5 Maximal binding capacity, nonspecific binding, and blank HAP binding

The maximal binding capacity of all ^{125}I-EGF preparations, including the different HPLC fractions, was investigated under the conditions as described in materials and methods by incubating an increasing number of receptors with a fixed concentration of labeled EGF. Irrespective of the iodination procedure used, the maximal binding capacity was >95%. The specific radioactivity, therefore, was not corrected for the binding capacity of the ^{125}I-EGF preparations.
For all EGF tracers prepared with the six different methods (n=35), the nonspecific binding ranged from 1 to 2% of the total counts added and the non-specific adsorption to HAP (blank HAP binding) ranged from 0.6 to 1.7%.

4 3 6 Effect of storage conditions of ^{125}I-EGF on tracer characteristics

During storage of ^{125}I-EGF preparations the nonspecific binding and the blank HAP binding of the tracer in binding experiments increased in a time- and temperature-dependent manner (Table 4 2) Irrespective of the iodination method used for iodination, at -20 °C and -80 °C, this increase was on average about 0 05% per day of storage, whereas at 4 °C, it was almost negligible Sephadex gelpermeation chromatography revealed that the increase in nonspecific/blank HAP binding did not parallel the release of ^{125}I from the ^{125}I-EGF, but did roughly parallel the appearance of a radioactive high molecular weight (^{125}I-HMW) component in the ^{125}I-EGF preparation (Table 4 2 and Fig 4 10)
The binding of the radioactive markers (see legend to Table 4 2) to HAP was compared to the amount of binding of the radioactivity in the column fractions after gelpermeation chromatography 70% to 80% of ^{125}I-BSA bound to the HAP The ^{125}I-HMW component also bound for 70% to the HAP The binding of both ^{125}I-EGF and ^{125}I to HAP was very low (below 1%) (Fig 4 10b)
According to these data, storage of ^{125}I-EGF at 4 °C seemed to be most convenient However, storage of ^{125}I-EGF at 4 °C resulted in nonequivalent tracer preparations within two weeks as observed by LEA (data not shown) In contrast, storage at -20 °C did not change the affinity of the ^{125}I-EGF preparation for at least up to two months Correct receptor binding data were obtained with these ^{125}I-EGF preparations if radioactive decay was taken into account only for the determination of the actual specific radioactivity

Table 4.2. *Characteristics of an Enzymobeads ^{125}I-EGF preparation during prolonged storage (42 days) at various temperatures.*

	NSB (%/day)[1]	blank HAP (%/day)	Free ^{125}I (%/day)	^{125}I-HMW (%/day)
+20 °C	nd[2]	0 059	0 85	0 012
+ 4 °C	0 018	0 018	0 48	0 020
- 20 °C	0 054	0 064	0 31	0 064
- 80 °C	nd	0 056	0 36	0 036

[1]per cent increase per day of storage, [2]not determined
Nonspecific binding and blank HAP were determined as described in materials and methods ^{125}I-HMW and free ^{125}I were determined by gelpermeation chromatography (Fig 4 10a) as percentage of the total radioactivity eluted from the column Approximately 5 x 10^6 cpm of ^{125}I-EGF were loaded on top of a 30 x 1 cm Sephadex G10/G50 column (5 cm of G10, 25 cm of G50) and eluted with PBB-buffer Fractions (10 dr) were collected and counted for radioactivity Carrier-free ^{125}I, ^{125}I-BSA (100 µg of BSA, ICI method), and freshly prepared ^{125}I-EGF were used as markers

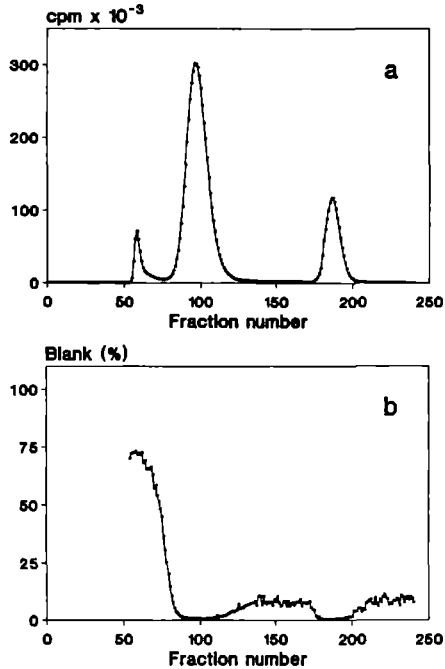

Fig. 4.10 a, Sephadex gelpermeation chromatography profile of an Enzymobeads-^{125}I-EGF, stored at -20 °C for 80 days; b, blank HAP binding of the radioactive moieties in the column fractions to 100 µl of HAP suspension.

4.4 Discussion

Binding studies of EGFR require an ^{125}I-EGF preparation of which the binding behavior towards the receptor is identical to that of unlabeled EGF (Kermode 1988). Moreover, an actual and accurate value for the specific radioactivity of the radioiodinated EGF is required (Munson 1983). In the present study, we compared six direct methods for radioiodination of mouse EGF. The binding behavior of the different ^{125}I-EGF preparations, as compared with that of unlabeled EGF, was investigated by comparison of saturation (increasing concentrations of ^{125}I-EGF) and competitive (fixed concentration of ^{125}I-EGF and increasing concentrations of unlabeled EGF) binding data. This comparison of saturation and competition binding data is generally confusingly designated as self-displacement analysis (Morris 1976, Calvo *et al.* 1983). We prefer the term Ligand Equivalence Analysis (LEA). With LEA, an accurate determination of the specific radioactivity can be performed when the apparent affinity of labeled and unlabeled ligand towards the receptor is identical. If not, the LE plot shows a line of which the slope differs from zero (Hollemans and Touber 1974,

Hollemans and Bertina 1975, Van Zoelen 1992).
The binding behavior of EGF preparations labeled with Chloramine T to HPM containing EGFR was studied. Apparently, the affinity of this radioligand was less than the affinity of unlabeled EGF. This nonequivalence between labeled and unlabeled EGF precludes an accurate determination of the specific radioactivity by LEA. Moreover, the LE plot in Fig. 4.1c showed curvilinearity which might be the result of heterogeneity of binding sites (Hollemans and Touber 1974) or of ligand.
There are other approaches besides LEA for determination of the specific radioactivity. In many studies the specific radioactivity of the radioligand is measured simply by determining the mass of the ligand and the corresponding radioactivity. The use of specfic radioactivity obtained in this way, in concert with nonequivalence in binding behavior between labeled and unlabeled ligand, has important consequences in binding studies. For a tracer with a lower affinity than unlabeled EGF, displacement analysis will result in an underestimated EGFR value, whereas for a tracer with a higher affinity, an overestimation of the receptor number will be obtained. In contrast, saturation analysis will result in correct values for the number of binding sites, but the apparent dissociation constant will be incorrect to an extent that depends on the affinity of the tracer compared with that of unlabeled EGF.
Iodogen and Iodo-beads have been claimed to be mild iodination reagents, compared to Chloramine T, because of their insolubility in water (Fraker and Speck 1978, Salacinski *et al.* 1980, Markwell 1982). Our results however, do not support this claim as far as iodination of EGF is concerned. The decrease in receptor-binding affinity of EGF labeled with Chloramine T, Iodogen or Iodo-beads is very likely due to the oxidation of readily oxidizable amino acid residues in the EGF molecule-binding region (e.g. methionine and amino acids with aromatic side chains). Indeed, methionine is readily oxidized by Chloramine T treatment (Stagg *et al.* 1970). The oxidative capacity is probably not due to the iodination reagent itself, but more likely to oxidative intermediates formed in these procedures. When dissolved in water, Chloramine T, the n-monochloro derivate of p-toluenesulfonamide, generates hypochlorous acid (HOCl), a soluble and strongly oxidative molecule. Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril), a virtually insoluble complex with four Chloramine T-like groups (Fraker and Speck 1978, Salacinski 1980), and Iodo-beads, Chloramine T molecules coupled onto polystyrene beads (Markwell 1982), also generate HOCl as oxidative intermediate in the iodination reaction. Comparable oxidation damage to EGF therefore likely occurs when either one of these methods is used. The oxidation of EGF by HOCl from Chloramine T, Iodogen, and Iodo-beads obviously has serious implications for the binding behavior of the labeled ligand. The mechanism of the remaining three methods studied involves other intermediates to generate a reactive iodine molecule. ICl acts via isotopic exchange (Contreras *et al.* 1983). In the conventional lactoperoxidase method, a relatively high amount of hydrogen peroxide, which could be harmful for the peptide, is added to initiate the lactoperoxidase reaction (Carpenter *et al.* 1975). According to the manufacturer, Enzymobeads initiate the iodination reaction mildly by a very slow generation of hydrogen peroxide after conversion of β-D-glucose by glucose oxidase. Unfortunately, we do not know the mechanism of Protag-125 iodination, but it is likely to act differently from Chloramine T iodination. Iodination of EGF with ICl, Protag-125, or Enzymobeads apparently does not affect the binding characteristics of EGF. This equivalence in binding behavior between labeled and unlabeled EGF suggests that none of these iodination methods results in

substantial damage to the protein, at least in relation to its interaction with EGFR. Perhaps the tyrosine residues favored for iodination in these methods are not in the region of the protein moiety involved in the receptor interaction. The unaltered binding behavior of these EGF preparations enables accurate measurements of the specific radioactivity. Moreover, a reliable estimation of EGF receptors by multiple-point Scatchard analysis can be established with respect to the apparent number of binding sites and apparent dissociation constants, both for saturation and competitive binding analyses.

All six methods used in the present study for direct radioiodination of EGF, generated highly heterogeneous ^{125}I-EGF preparations. These results are in agreement with studies by Matrisian *et al.* who showed that radioiodination of EGF by the Chloramine T method results in a heterogeneous ^{125}I-EGF preparation (Matrisian *et al.* 1985). The heterogeneity may be the result of the presence of five tyrosine residues in the EGF molecule (Savage *et al.* 1972), which provides the possibility for various labeled EGF species to be formed during iodination.

In the Chloramine T, Iodogen and Iodo-beads iodination reactions, nearly all of the iodinated products had shorter HPLC retention times than unlabeled EGF. Because oxidation of methionine-containing proteins decreased their retention time on reversed-phase HPLC (Seidah *et al.* 1980), it may be concluded from these observations that EGF is oxidized during these iodination reactions. One of the effects of oxidation of ^{125}I-EGF is the formation of covalent-like ligand-receptor complexes (Comens *et al.* 1982), which could affect equilibrium binding measurements. However, another study indicated that the covalent-like binding properties are not affected by various methods for iodination of EGF. Furthermore, even unlabeled EGF formed this covalent-like ligand-receptor complex (Marti *et al.* 1989). Thus, it is doubtful whether the forms of ^{125}I-EGF iodinated by different methods are distinguishable as far as their covalent-like binding properties are concerned.

LEA of different ^{125}I-EGF moieties after HPLC purification of Chloramine T-^{125}I-EGF revealed that none of these moieties showed ligand equivalence. Therefore, the quality of a ^{125}I-EGF preparation with respect to its equivalence to unlabeled EGF in receptor-binding characteristics cannot solely be determined by its purity and retention time on HPLC. HPLC purified Chloramine T-^{125}I-EGF preparations should therefore also not be used in receptor competition assays, using a single amount of ^{125}I-EGF and increasing concentrations of unlabeled EGF. On the other hand, the radioligands in the pooled HPLC fractions of Enzymobeads-^{125}I-EGF all showed equivalence in binding behavior between labeled and unlabeled ligand, except for the E1 tracer (Fig. 4.8). The EGF protein in this fraction could have been present already in the unlabeled EGF preparation as a probably oxidized contaminant. However, this small amount of E1 tracer did not introduce nonequivalence in binding behavior between the overall Enzymobeads-^{125}I-EGF preparation and unlabeled EGF. It is therefore concluded that it is not essential to purify an Enzymobeads-labeled EGF preparation to homogeneity to obtain a ^{125}I-EGF preparation with ligand equivalence. HPLC purification is also unnecessary when EGF is radioiodinated by using ICl or Protag-125, because these ^{125}I-EGF preparations show ligand equivalence.

The observation that the maximal binding capacity of all tracers is similar and is still >95%, is an indication that neither cysteine residues or other amino acid residues critical for receptor binding are affected by iodination and/or oxidation, and that the oxidation of amino acid residues does not prevent, but only alters the ligand receptor

interaction. It is clearly shown, in agreement with Kermode (1988) that maximal bindability and equivalence in binding behavior between labeled and unlabeled ligand are two separate aspects of radioligand quality, which both have to be examined before the radioligand is used in receptor studies. Moreover, treatment of EGF in the presence of nonradioactive NaI with ICI (iodination) or with Chloramine T (iodination and oxidation) did not affect its biological activity significantly. This was established in a serum-free proliferation assay with normal rat kidney cells (Van Zoelen *et al.* 1988). These results also indicate that amino acids critical for receptor binding are not affected by either iodination or oxidation. Similar observations were made by Matrisian *et al.* (1985), who showed that EGF radioiodinated with Chloramine T retained its biological activity.

Prolonged storage at -20 °C did not affect the receptor-binding properties of the ^{125}I-EGF preparations. However, an ^{125}I-HMW that included probably an iodinated form of BSA which increased nonspecific binding, and free ^{125}I increased during storage of ^{125}I-EGF at -20 °C and -80 °C. Nevertheless, correct receptor binding data were obtained if the specific radioactivity was corrected for radioactive decay only. For routine use, it was decided to store ^{125}I-EGF preparations at -20 °C and to prepare ^{125}I-EGF freshly at least every four weeks.

Our present findings show that Chloramine T, Iodogen, or Iodo-beads iodination affects the ligand-receptor interaction, resulting in a lesser receptor binding affinity than that of unlabeled EGF. As a consequence, such ligands can not be used for reliable measurements of EGF receptors. This holds particularly for those assays in which Scatchard analyses are performed by using a single amount of ^{125}I-EGF and increasing concentrations of unlabeled EGF. HPLC purification of ^{125}I-EGF, prepared by harsh oxidative iodination methods, also does not result in an EGF tracer with better binding characteristics than the overall ^{125}I-EGF preparation. In contrast, ^{125}I-EGF preparations with binding affinity towards EGFR identical to the affinity of the unlabeled growth factor could be prepared by using ICI, Protag-125, or Enzymobeads. Sufficiently high specific radioactivities could be obtained with these methods. Therefore, sensitive and more-reliable EGFR assays can be performed by using EGF radioiodinated by these reagents. Moreover, HPLC purification of ^{125}I-EGF prepared by these methods is considered superfluous, because the overall heterogeneous ^{125}I-EGF preparation shows ligand equivalence. HPLC purification, however, can yield tracers of very high specific radioactivity tracers (>3000 Ci/mmol) and ligand equivalence through the use of these mild radioiodination methods.

References are listed on pages 99-110.

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CHAPTER 5

ASSAYS FOR EPIDERMAL GROWTH FACTOR RECEPTOR LIGAND(S) IN MAMMARY TUMOR CYTOSOLS

5.1 Introduction

As described in previous chapters, numerous studies have been reported regarding the epidermal growth factor receptor (EGFR) in human breast cancer. In contrast, the attention paid to the assessment of the ligand(s) for this receptor has been nearly negligible and is limited to a few studies (Macias *et al.* 1989, Foekens *et al.* 1989b). Assessment of EGFR ligand(s) is possible by using an EGFR-containing preparation as the binding source (radioreceptor assay, RRA). However, apart from EGF (Carpenter and Cohen 1990), transforming growth factor alpha (TGFα, Todaro *et al.* 1980), vaccinia growth factor (VGF, Stroobant *et al.* 1985), amphiregulin (Shoyab *et al.* 1985), and cripto (Ciardiello *et al.* 1991) have been shown to bind and activate the EGF receptor. Therefore, an RRA determines an overall concentration of EGFR ligand(s). A human placental membrane (HPM) preparation was used as the binding source in the RRA and ^{125}I-mouse EGF as the tracer. EGFR ligand(s) were assessed in cytosols derived from human breast tumors and mouse mammary tumors. In addition, a specific polyclonal antibody against mouse EGF (mEGF) was raised and enabled us to develop a sensitive and specific radioimmunoassay (RIA) for mEGF in mouse mammary tumor cytosols. The development and performance of both the RRA and RIA procedures are described in this chapter.

5.3 Materials and methods

5.3.1 Materials

Receptor grade mouse EGF (mEGF) was obtained from Bioproducts for Science, Inc. (Indianapolis, IN, U.S.A.). Human recombinant EGF (hEGF) was obtained from Bioproducts for Science or from Amersham International (Amersham, Buckinghamshire, U.K.). Human recombinant TGFα (hTGFα) was obtained from PeproTech Inc. (Rocky Hill, NJ, U.S.A.). Carrier-free Na^{125}I was obtained from Amersham International. Iodine monochloride (ICl) was obtained from Merck (Darmstadt, Germany). Enzymobeads and hydroxylapatite (HAP; DNA grade Biogel HTP) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). β-D-Glucose was supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bacitracin was obtained from Serva (Heidelberg, Germany). Bovine serum albumin (BSA) was purchased from Behringwerke AG (Marburg/Lahn, Germany).

5.3.2 Tissue cytosols

Tumor tissue was pulverized in the frozen state by means of a microdismembrator (Braun, Melsungen, Germany). The tissue powder was homogenized in five volumes of buffer A (10 mM K_2HPO_4/KH_2PO_4, 1.5 mM K_2EDTA, 10 mM monothioglycerol, 3 mM NaN_3, and 10%, v/v, glycerol, pH 7.4). After centrifugation for 10 min at 800 x g (4 °C), the supernate was further centrifuged for 1 h at 100,000 x g (4 °C). The supernate was referred to as cytosol and used for measurement of EGFR binding factors.

EGFR ligand(s)-free cytosols, which were used in the standard curves, were obtained from human uterus or breast tumors. Tissue was grinded with a Retsch ultra centrifugal mill (Type ZM1, Retsch, Haan, Germany) and homogenized in ice-cold phosphate buffer A without glycerol (5 ml per gram of tissue). The homogenate was centrifuged for 1 h at 45,000 x g (4 °C). 5 ml-aliquots of the supernate were lyophilized and stored at 4 °C. Before use, a lyophilized cytosol specimen was reconstituted with 5 ml of water and incubated with 800 mg of charcoal (Norit A, Fisher Scientific, U.S.A.) to adsorb possibly interfering substances (EGF and related peptides).
A mEGF-containing cytosol was obtained from mouse submaxillary glands. Glandular tissue (about 50 mg) was homogenized in 5 ml of buffer A using an Omni-1000 (Omni International, Waterbury, CT, USA) for 10 sec at 4 °C. The homogenate was centrifuged for 1 h at 100,000 x g (4 °C).

5.3.3 RRA procedure

Approximately 15,000 cpm of ^{125}I-mEGF (10 µl, 500 Ci/mmol) and 50 µl of tumor cytosol were equilibrated in duplicate incubations with 100 µl of HPM (containing 50 fmol EGFR protein, prepared as described in chapter 2), in a final volume of 170 µl of assay buffer (20 mM phosphate, 150 mM NaCl, 50 µM bacitracin, 0.1% BSA, pH 7.4) for 16-20 h at 20 °C. In the standard curve ^{125}I-EGF (10 µl) was incubated with 50 µl of charcoal-treated cytosol, 10 µl of EGF standard, and 100 µl of HPM. The standard curve was prepared using a charcoal-treated human breast tumor or human uterus cytosol to correct for matrix effects. Separation of receptor-bound from free ligand was performed using the hydroxylapatite (HAP) procedure. Results were calculated using a standard curve-fitting RIA program.

5.3.4. RIA procedure

An antibody was raised against mEGF in rabbit(s). The mEGF was coupled to BSA by the glutaraldehyde method (Avrameas and Ternynck 1971). 0.5 ml of a solution of the immunogen was mixed with an equal volume of Freund's complete adjuvans. Booster injections were given monthly (3 times).
The RIA procedure was performed as described for the RRA procedure, except that 100 µl of a 1:40,000 dilution of the antiserum was used in stead of HPM. For separation of antibody-bound from free ^{125}I-EGF we again used the HAP procedure.

5.3 Results

5.3.1. RRA

A binding competition assay (radioreceptor assay, RRA) involving the HAP procedure for separation of receptor-bound from free ^{125}I-EGF was introduced using HPM as EGF binding source.

First, the appropriate dilution of the HPM preparation was determined. A membrane dilution curve was prepared in assay buffer and samples were incubated with a fixed amount of ^{125}I-mEGF (15,000 cpm). At a 40-fold dilution of HPM, 40-50% of the ^{125}I-EGF added was bound (Fig. 5.1a), and this preparation was used in the RRA.

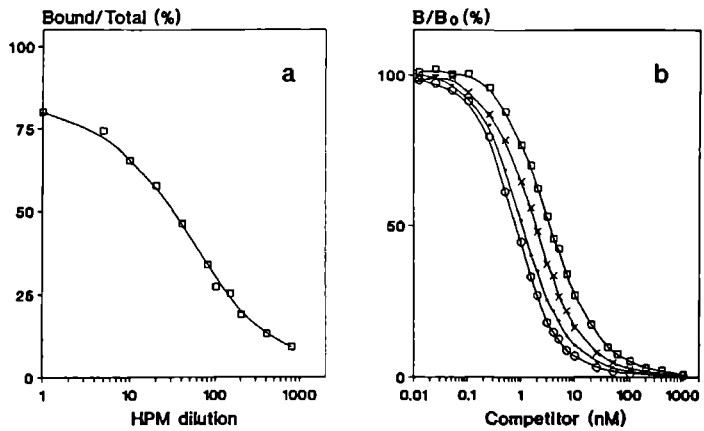

Fig. 5.1 a, HPM dilution curve obtained with 15,000 cpm ^{125}I-EGF. ^{125}I-EGF bound to diluted HPM preparations was determined by adsorption of the bound fraction to HAP (see chapter 2) and counting of the HAP pellet after two washing steps. b, Competition curves of hEGF (two different preparations), mEGF, and hTGFα using a 10-fold diluted HPM preparation. About 15,000 cpm ^{125}I-mEGF and increasing concentrations of competitor were incubated with 100 µl of the HPM preperation. (•), mEGF; (o), hEGF (Amersham Int.); (x), hEGF (Bioproducts); (□), hTGFα.

It was assumed that the quantity of the growth factor preparations was correctly stated on the vials. Stock solutions were made by dissolving the entire vial contents in 0.05 M phosphate buffer (pH 7.4). Competitive binding curves of hEGF (two different preparations), mEGF, and TGFα are shown in Fig. 5.1b. It is obvious that the competition of ^{125}I-EGF by hTGFα is less as compared to either hEGF (25% and 57%) or mEGF (33%). Apparently, hEGF obtained from different manufacturers exhibited different receptor binding properties. As long as there are no standard EGF and TGFα preparations, the differences observed in Fig. 5.1 may also have been resulted from differences in concentrations. These results imply that dissimilar assay results are introduced if different EGF preparations are used as standard in the RRA. We chose hEGF (Amersham) as standard if human tumor cytosols were assayed and mEGF if cytosols derived from mouse mammary tumors were analyzed for EGFR ligand(s).

Table 5.1 Reproducibility of the RRA

		EGFR ligand(s) mean ± S.D. (fmol/50 μl)	C.V. (%)
Within-assay variation	n=10	8.1 ± 0.6	7.8
	n=10	39.2 ± 3.0	7.7
	n=10	184.3 ± 11.2	6.1
Between-assay variation	n=5	8.2 ± 1.6	19.6
	n=5	36.3 ± 2.2	6.0
	n=5	198.5 ± 5.3	2.7

Three mouse mammary tumor cytosols were analyzed in 10 duplicate experiments to assess the within-assay variation. The between-assay variation was examined by measuring EGFR ligand(s) in the three cytosols in five separate experiments.

The reproducibility of the RRA was investigated using three mouse mammary tumor cytosols with different levels of EGFR ligand(s). The results of this examination of the within- and between-assay variation are shown in Table 5.1. The within-assay variation was found to be about 7%, whereas the between-assay variation ranged from 2.7% to 19.6%.

The sensitivity of the RRA was evaluated by an experiment in which a pool of human breast tumor cytosols was serially diluted with charcoal-treated cytosol, which by earlier determination was found to be devoid of EGFR ligand(s). The hEGF standard RRA curve obtained in this experiment is shown in Fig. 5.2a. The concentration of EGFR ligand(s) in the diluted cytosols were linearly related to the corresponding dilution factor, down to about 3 fmol of EGF-activity per assay tube (=3 fmol/50 μl of cytosol) (Fig. 5.2b).

A recovery experiment was performed in which a human breast tumor cytosol pool was assayed for EGFR ligand(s) employing standard curves prepared in different matrices (buffer and charcoal-treated cytosols). From Table 5.2 it appears that the composition of the medium in which the standard curve is prepared is of great influence on the values obtained. Lower values of EGFR ligand(s) were obtained using the standard curve prepared in the charcoal-treated cytosols as compared to the values obtained using the standard curve prepared in buffer. Furthermore, the tumor cytosol was supplemented with fixed amounts of hEGF (20, 40, and 100 fmol) to examine whether the hEGF added could be recovered. Using the standard curve prepared in charcoal-treated cytosol of either human breast tumor or human uterus, the recovery of hEGF was quantitative (102% and 98% for charcoal-treated breast tumor cytosol and uterus cytosol respectively). Using the standard curve prepared in buffer the measured values exceeded the expected values (recovery 116%).

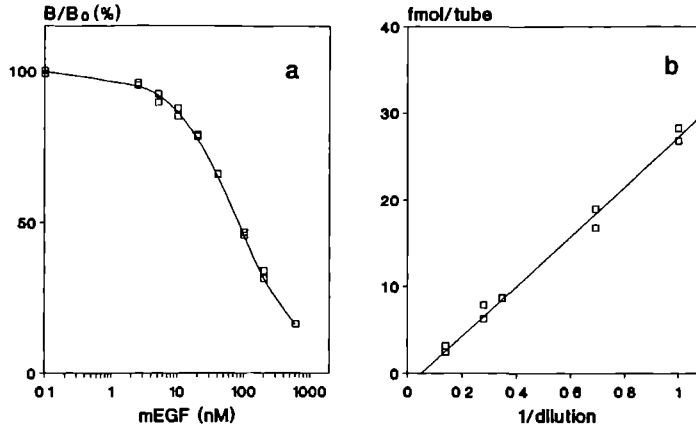

Fig. 5.2. a, Standard curve of hEGF (Amersham) obtained by incubation of about 15,000 cpm ^{125}I-mEGF and increasing concentrations of hEGF with 100 µl of the 40-fold diluted HPM preparation b, Levels of EGFR ligand(s) in a pool of human breast tumor cytosols serially diluted with charcoal-treated cytosol.

***Table 5.2** Recovery of hEGF added to cytosol*

Standard curve prepared in:	Buffer		Breast tumor cytosol		Uterus cytosol	
	Expected (fmol)	Found (fmol)	Expected (fmol)	Found (fmol)	Expected (fmol)	Found (fmol)
EGF added to human breast tumor cytosol (fmol)						
0		23.8		13.9		13.1
20	43.8	47.1	33.9	33.7	33.1	32.0
40	63.8	72.5	53.9	56.8	53.1	54.7
100	123.8	132.8	113.9	113.7	113.1	111.3

A human breast tumor cytosol pool was analyzed for EGFR ligand(s), in the absence or presence of added hEGF, using standard curves prepared in buffer or in charcoal-treated human breast tumor c q. human uterus cytosol.

5.3.2. RIA

A specific radioimmunoassay (RIA) for mEGF was developed using a polyclonal rabbit anti-mEGF antibody which was obtained from rabbits by immunization with the receptor grade mEGF. The RIA also involved HAP to separate antibody-bound and free ^{125}I-mEGF. The antiserum dilution curve is shown in Fig 5.3a. The antiserum working dilution used was 1:40,000, yielding a bound/total ratio of about 40%.
The specificity of the antiserum was assessed by measuring the binding ability of mEGF, hEGF (two different preparations) and hTGFα to the antibody. As far as these ligands are concerned, the RIA was highly specific for mEGF, since no cross-reactivity was observed with either hEGF or hTGFα (Fig. 5.3b).
The reproducibility of the RIA for the assay of mEGF was investigated using a mouse submaxillary gland cytosol (Table 5.3). The within-assay variation was 3.2-5.9%, and the between-assay variation was 6.1-8.5%.

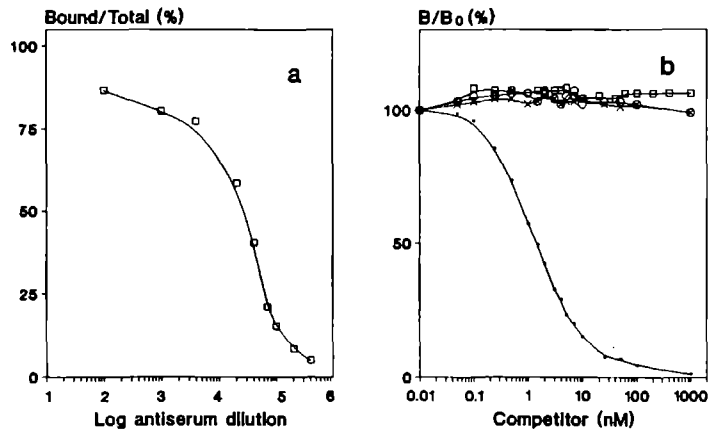

Fig. 5.3 a, Antiserum dilution curve obtained with approximately 15,000 cpm ^{125}I-mEGF. Bound ^{125}I-mEGF was determined by adsorption to HAP (see chapter 2) and counting of the HAP pellet after two washing steps. b, Cross-reactivity of the anti-mouse EGF antiserum with mEGF, hEGF and hTGFα. About 15,000 cpm ^{125}I-mEGF was incubated together with increasing concentrations of competitors with 100 µl of a 1:40,000 diluted antiserum. (•), mEGF; (o), hEGF (Amersham Int.); (x), hEGF (Bioproducts); (□), hTGFα.

The sensitivity of the RIA was evaluated in an experiment in which EGF was measured in mouse submaxillary gland cytosol that was serially diluted with charcoal-treated cytosol. The mEGF standard curve obtained in this experiment is depicted in Fig. 5.4a. The concentrations of mEGF in the diluted cytosols were linearly related to the dilution factor (Fig. 5.4b) down to about 2 fmol of mEGF per assay tube (=2 fmol/50 µl of cytosol). This parallellism could also be observed in Table 5.3.

***Table 5.3** Reproducibility of the RIA*

		mEGF mean ± S.D. (fmol/50 μl)	C.V. (%)
Within-assay variation	n=10	93.7 ± 3 2	3.2
	n=10	45.2 ± 1.6	3.6
	n=10	24.0 ± 1.4	5.9
Between-assay variation	n=9	96.1 ± 5.9	6.1
	n=9	46 8 ± 4.0	8.5
	n=9	23.6 ± 1.9	8.1

A mouse submaxillary gland cytosol and 1 2 and 1 4 dilutions were analyzed for mEGF content by RIA in 10 duplicate experiments for examination or the within-assay variation The within-assay variation was examined by measuring mEGF the cytosol in 9 separate experiments

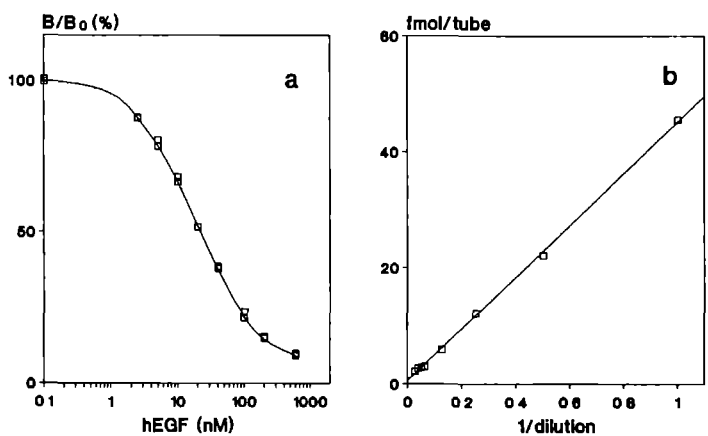

***Fig. 5.4.** a, Standard RIA curve of mEGF obtained by incubation of about 15,000 cpm ^{125}I-mEGF and increasing concentrations of mEGF with 100 μl of the 1 40,000 diluted antiserum b, Levels of mEGF in a mouse submaxillary gland cytosol serially diluted with charcoal-treated cytosol*

5.5 Discussion

It appears from the present chapter that EGFR ligand(s) can be reproducibly assessed in both human and mouse mammary tumor cytosols by a highly sensitive RRA. It is obvious that no specificity is obtained by this assay, because there are several growth factors that are able to compete with ^{125}I-mEGF for binding to the EGF receptor. Measurement of EGFR ligand(s) in human tissue cytosols require hEGF as a standard. It is important to realize that hEGF preparations from different manufacturers behave different in receptor binding studies. These differences in apparent affinity between the different hEGF preparations might be the result of amino acid substitutions in the respective recombinant EGF preparations. However, until now a standard preparation is lacking, which implies that the question can be raised whether the differences in behavior are based upon differences in affinity or merely result from falsely assumed concentrations. As a result, the level of EGFR ligand(s) obtained depends on the hEGF preparation used in the RRA. Furthermore, it is imperative to prepare the standard curve in a charcoal-treated cytosol (e.g. from human breast tumors or human uterus) to obtain proper parallellism.
The binding characteristics of EGF and TGFα are generally believed to be similar (Todaro *et al.* 1980, Massague *et al.* 1983). However, in some studies TGFα exhibits a lesser affinity to bind to the EGF receptor than does EGF (Winkler *et al.* 1989, Korc *et al.* 1991). In the present study we also observed differences in the relative binding affinities of hTGFα and hEGF towards the human placental EGF receptor. This difference in affinity between EGF and TGFα and the potential presence of other EGF-like peptides in breast tumor cytosols that interact with the EGF receptor, precludes determination of precise molar concentrations of the EGFR ligand(s) present in any tumor cytosol by RRA. Moreover, no information regarding the nature of the ligand(s) present can be provided. Therefore, the availability of specific antibodies and highly sensitive radioimmunoassays to measure the levels of the individual growth factors in human breast tumor cytosols is highly desirable. However, specific antibodies to all EGFR ligand(s) are not available and the availability of breast tumor cytosol is limited. Therefore, it was considered worthwile to measure the overall levels of EGFR ligand(s) in human and mouse mammary tumor cytosols by RRA.
Pilot studies were designed to examine the associations between EGFR ligand(s) and EGFR, and steroid hormone receptors in human breast cancer and in a mouse mammary tumor model. For the study on EGFR ligand(s) in human breast cancer the reader is referred to the doctoral thesis by P.G. Koenders: "Epidermal growth factor receptor and its ligand(s): associations with prognosis of patients with breast cancer", University of Nijmegen, 1992. The examination of EGFR ligand(s) in cytosols derived from mouse mammary tumors is described in the next chapter. In the mouse tumor cytosols it was also possible to assess specifically EGF levels with high sensitivity and reliability in the tumor cytosols using the highly specific RIA.

References are listed on pages 99-110.

CHAPTER 6

EPIDERMAL GROWTH FACTOR RECEPTOR AND ITS LIGAND(S) IN MOUSE MAMMARY TUMORS DURING PROGRESSION FROM HORMONE DEPENDENCE TO HORMONE INDEPENDENCE

6.1 Introduction

It has long been recognized that human breast tumors which at first respond to hormonal therapy (e.g. with anti-estrogens) later develop resistance to this treatment. Progression to hormone resistance is accompanied by a loss in detectable ER and PgR in the tumors (Jensen *et al.* 1982). A progression of hormone dependence (HD) to hormone independence (HI) is also observed in serial transplants of tumors that are induced in ovariectomized female GR strain mice by continuous administration of estrone and progesterone (Sluyser and Van Nie 1974, Sluyser *et al.* 1976). The induced tumors are initially HD, but during transplantation they become hormone responsive (HR) and finally HI.
Factors that regulate growth and development of mammary tissue include EGF and related peptides (e.g. transforming growth factor-α, TGF-α) (Dickson and Lippman 1987, Dickson 1990). EGF and EGF-like peptides are potent mitogens that elicit their effect by binding to a 170,000 Da transmembrane glycoprotein (EGFR), which possesses an intrinsic tyrosine kinase domain believed to be involved in the transduction of the mitogenic signal (Hock and Hollenberg 1980, Downward *et al.* 1984, Yarden and Schlessinger 1985, Gill *et al.* 1988). Studies in human breast carcinomas established an inverse relationship between ER and EGFR and suggested EGFR to be associated with the resistance to endocrine treatment of advanced breast cancer (Sainsbury *et al.* 1987, reviewed by Klijn *et al.* 1992). In order to investigate this in more detail, we have now analyzed GR mouse mammary tumors which progress from HD to HI. Tumors obtained from four different lines of this mammary tumor model (HD, HR and HI transplants) were analyzed for EGFR and EGFR ligand(s) to examine if EGFR and/or its ligand(s) are related to or involved in the progression of breast tumors to hormone independence in this animal model.

6.2 Materials and methods

6.2.1 Materials

Receptor grade mouse EGF (mEGF) was obtained from Bioproducts for Science (Indianapolis, IN, U.S.A.). ^{3}H-labeled estradiol, ^{3}H-org2058, and carrier free Na^{125}I were obtained from Amersham International (Amersham, Bucks., U.K.). Estrone and progesterone were from N.V. Organon Co. (Oss, The Netherlands). Enzymobeads and hydroxylapatite (DNA grade Biogel HTP, HAP) were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). ß-D-Glucose was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Bacitracin was obtained from Serva (Heidelberg, Germany). Bovine serum albumin (BSA) was purchased from Behringwerke AG (Marburg, Germany).

6.2.2 Tumor initiation and transplantation

Mouse mammary tumors were obtained from Dr. Sluyser (The Netherlands Cancer Institute). The tumors had been induced in female mice from the GR strain by estrone and progesterone treatment. Established tumors were transplanted in the flanks of

castrated O20 x GR F_1 mice as described elsewhere (Sluyser and Van Nie 1974). For testing of hormone dependence, tumor tissue was inoculated into 2 or more castrated female or male mice that received no hormone treatment. Two or more castrated animals, treated with estrone and progesterone as above, served as controls. The animals were checked regularly for 3 months. If within this period no tumor appeared in the animals that did not receive hormones, but tumors grew progressively in 1 or both of the treated animals, the tumor tested was considered HD. However, if the tumor grew in the nontreated animals as well, and the time of tumor appearance was equal to that in the treated group, the tumor was considered HI. Tumors that were transplantable into nontreated animals but appeared more than 1 week earlier in the hormone-treated animals were called HR. HD tumors were transplanted every 4 to 6 weeks; HI tumors were transplanted every 2 to 4 weeks (Sluyser and Van Nie 1974).

6.2.3 Tumor cytosols and membrane fractions

400-600 mg of mouse mammary tumor tissue stored at -80 °C, was pulverized in the frozen state by means of a dismembrator (Braun, Melsungen, Germany). The tissue powder was homogenized in five volumes of assay buffer (0.01 M phosphate buffer pH 7.4, containing 0.01 M K_2HPO_4/KH_2PO_4, 0.0015 M dipotassium EDTA, 0.003 M NaN_3, 0.01 M monothioglycerol, and 10%, v/v, glycerol). After centrifugation for 10 min at 800 x g (4 °C), the supernatant was further centrifuged for 1 h at 105,000 x g (4 °C). The resulting supernatant was referred to as cytosol and was used for measurements of steroid hormone receptors, EGF and EGFR ligand(s). The 105,000 x g pellet was rinsed with 1 ml of EGFR assay buffer (0.02 M phosphate buffer, pH 7.4, containing 0.15 M NaCl and 50 μM bacitracin). Subsequently the pellet was resuspended in 2 ml of EGFR assay buffer. This fraction was referred to as membrane fraction and was used for the determination of EGFR concentration.

6.2.4 Protein assays

Cytosolic protein was measured using the Folin-Ciocalteus reagent (Lowry *et al.* 1951). Membrane protein was assessed by the Coomassie brilliant blue assay method (Bradford 1976). In both protein assays human albumin was used as a standard.

6.3.5 Receptor assays

Steroid hormone receptors were measured by the dextran-coated charcoal (DCC) method as recommended by the EORTC (1980). In short, 50 μl aliquots of tumor cytosol were incubated with increasing concentrations of ^{3}H-estradiol or ^{3}H-Org2058 in a final volume of 60 μl of assay buffer for 16 h at 4 °C. Separation of receptor-bound from free ligand was performed by the addition of 100 μl of DCC suspension and incubation for another 10 min at 4 °C. After centrifugation for 15 min at 800 x g (4 °C), 100 μl of the supernate was counted for radioactivity in a scintillation counter. EGFR was measured using the HAP assay as described in chapter 2. EGF was iodinated using Enzymobeads, to a specific radioactivity of about 500 Ci/mmol.

6.3.6 EGF radioreceptorassay/radioimmunoassay

For the assessment of EGFR ligand(s) by radioreceptor assay (RRA), 50 μl of mouse tumor cytosol and about 15000 counts/min of ^{125}I-mEGF were incubated with a human placental membrane preparation (HPM) containing about 500 fmoles of EGFR protein/ml, as described in the previous chapter.
mEGF was measured in the tumor cytosols specifically using a rabbit anti-mouse EGF antibody (working dilution 1:40,000). A mouse submaxillary gland cytosol served as a positive control for mEGF in the radioimmunoassay (RIA).

6.3.7 Statistics

Associations between variables were assessed by the Spearman rank correlation test. Homogeneity between groups was tested nonparametrically by means of the Wilcoxon two-sample test (Kruskall-Wallis test for multiple groups). Test results were considered significant at the $P<0.05$ level. Calculations were performed by using SAS (Statistical Analyzing System) statistical software (SAS Institute Inc, Cary, NC, USA).

6.4 Results

6.4.1 Steroid hormone receptors

ER and PgR levels in the HI tumor cytosols were significantly lower as compared to those in HD and HR tumors ($P=0.004$ and $P=0.001$ respectively, Fig. 6.1a and 6.1b). HI tumors contained only 30% of the ER concentration that was found in HD and HR tumors (Fig. 6.1a) and there was a complete lack of detectable PgR in HI tumors (Fig. 6.1b). These results are similar to those observed earlier using the same mouse tumor model (Sluyser and Van Nie 1974, Moncharmont *et al.* 1991).

6.4.2 EGFR

All 26 GR tumor samples contained measurable amounts of EGFR protein, total levels ranging from 8 to 614 fmol/mg of membrane protein (Kd range 0.5 to 2.5 nM). An inverse correlation between EGFR and ER ($R_s=-0.463$, $P=0.02$) was observed, while there was no significant relationship between EGFR and PgR ($R_s=-0.298$, $P=0.14$). EGFR levels differed significantly between the HD, HR, and HI subgroups ($P=0.006$). Mean EGFR levels in HI tumors were 2.5 to 3-fold higher than they were in HD tumors (Fig. 6.2a).

6.4.3 EGFR ligand(s)

All GR mammary tumor cytosols contained EGFR ligand(s) (range: 20 to 723 fmol/mg of cytosolic protein). The level of EGFR ligand(s) was found to be positively associated

with ER (R_s=0.532, P=0.005). Also a positive association was found between EGFR ligand(s) and PgR, although not statistically significant (R_s=0.249, P=0.22). Fig. 6.2b shows that the mean level of EGFR ligand(s) in HD tumors was about three-fold higher than in HI tumors (P=0.004). The level of EGFR ligand(s) was inversely correlated with EGFR (R_s=-0.578, P=0.002) in the tumors. In contrast to the radioreceptor assay, when using the radioimmunoassay involving a rabbit anti-mouse EGF antibody, EGF levels in the tumor cytosols were negligible.

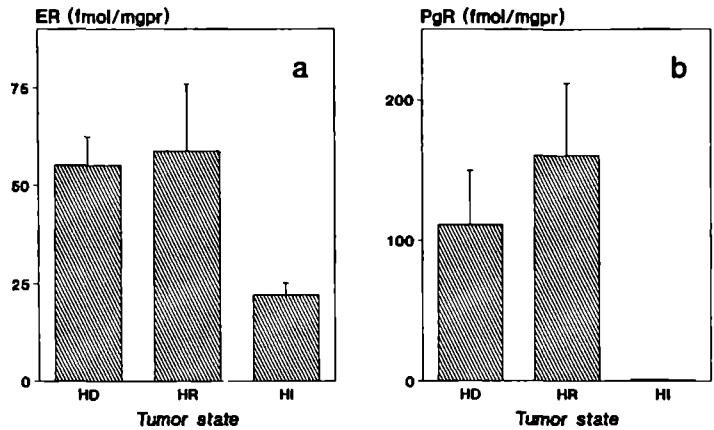

Fig. 6.1 Relation between the hormonal status of serially transplanted mouse mammary tumors (HD, HR, HI) versus cytosolic ER (a) and PgR levels (b). Steroid receptor levels are represented as fmol/mg of cytosolic protein ± SEM.

6.5 Discussion

The loss of hormone dependence of human breast cancer and the concomitant loss of steroid hormone receptors in the tumors are generally acknowledged. However, additional tumor differentiation markers are required for a better prediction of the patients' response to endocrine therapy. Studies reporting the assessment of EGFR in human primary breast tumor biopsies predominantly showed the higher EGFR levels in the ER negative subgroup of tumors, suggesting this biological parameter to provide such additional information (reviewed by Klijn *et al.* 1992).
In the present report we have measured EGFR and EGFR ligand(s) in the GR mouse mammary tumor model, in which the tumors gradually become HI during serial transplantation. Several HD, HR, and HI mouse mammary tumors were examined for EGFR and EGFR ligand(s), in addition to ER and PgR. The steroid hormone receptor levels decreased during progression to hormone independence. HI tumors still contained measurable amounts of ER, but no PgR. These results are similar to those obtained earlier using the same tumor model (Sluyser and Van Nie 1974, Moncharmont *et al.* 1991).

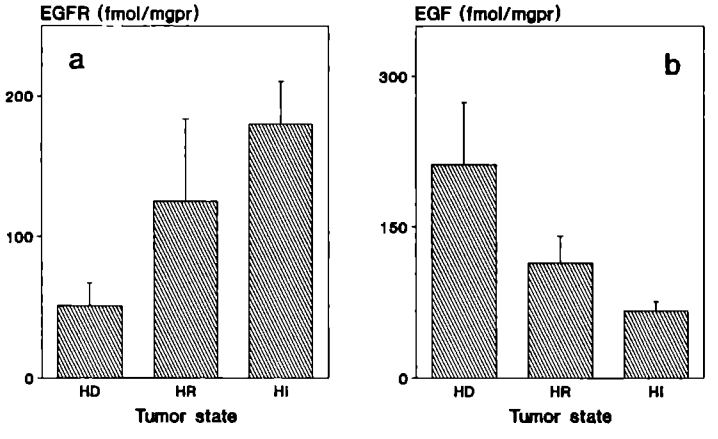

***Fig. 6.2** Relation between tumor status versus mean plasma membrane EGFR concentration (fmol/mg of membrane protein ± SEM) (a), and cytosolic EGFR ligand(s) (fmol/mg of cytosolic protein ± SEM) (b) in the tumors.*

EGFR increased during progression to hormone independence. This observation and the inverse association between ER and EGFR are in agreement with studies on ER and EGFR in human breast cancer, in which a higher sensitivity of EGFR positive tumors to growth promoting EGFR ligand(s) has been suggested to result in the poor prognosis of patients with those tumors (Sainsbury *et al.* 1987). In the present study, an inverse correlation was observed between EGFR and EGFR ligand(s), measured by using the radioreceptor assay, and a positive relationship between ER and EGFR ligand(s). Using the radioimmunoassay we could not detect EGF in the cytosols. The EGFR ligand(s) measured in the radioreceptor assay may therefore represent TGF-α. Nevertheless, the positive association between the level of EGFR ligand(s) and ER in this mouse tumor model suggests that the synthesis of EGFR ligand(s) could be regulated by estrogens, which is consistent with the finding that estrogens control the synthesis of EGF-like peptides in ER-positive human breast cancer cell lines (Dickson *et al.* 1986, Mori *et al.* 1986, Dickson and Lippman 1987).

Regarding the inverse relationship between EGFR and EGFR ligand(s), the receptor is known to become internalized upon ligand binding (downregulation) and the low levels of EGFR in the HD/HR tumors compared to HI tumors may be the result of downregulation of the receptor by its ligand(s) produced under ER control. In this view, during progression from HD to HI this downregulation is removed in the absence of ER induced EGFR ligand(s) resulting in elevated EGFR levels in the tumors.

The mice bearing HI tumors did not receive estrogen. From previous studies, however, it is known that treatment of such mice with estrogen does not result in an increase in the tumor PgR levels, indicating the presence of detectable but nonfunctional ER

(Moncharmont 1991). Therefore, assuming the synthesis of EGFR ligand(s) to be under ER control, administration of estrogen in these mice tumors also would not have been resulted in the synthesis of EGFR ligand(s) by these tumors. Therefore, it seems unlikely that the high levels of EGFR in HI tumors result from the fact that the mice bearing these HI tumors did not receive estrogen.

HI was associated with low levels of EGFR ligand(s). As a consequence, instinctively one would assume the lower levels of EGFR ligand(s) to be associated with bad prognosis. However, a study analyzing EGFR and its ligand(s) in human breast tumor biopsies performed in our laboratory, showed the higher levels of both EGFR and EGFR ligand(s) in human breast tumor cytosols to be associated with worse prognosis of patients (P.G. Koenders, thesis 1992). Our results show that the progression of tumors from HD to HI is associated with a decrease in ER and a concomitant increase in EGFR. Provided the positive relationship between ER and EGFR ligand(s), the increase in EGFR seems to be a secondary effect rather than a key event in the progression to hormone independence in this mouse mammary tumor model.

The present study is, to our knowledge, the first one regarding EGFR and its ligand(s) in an *in vivo* mammary tumor model and showed homology with studies regarding EGFR in human breast cancer. Therefore, this mouse mammary tumor model offers attractive possibilities to further examine the involvement of the EGF/TGFα-EGFR pathway and its interactions with the steroid hormone receptor pathway in the progression from hormone dependent to hormone independent breast cancer.

References are listed on pages 99-110.

CHAPTER 7

GENERAL DISCUSSION

It has long been recognized that estradiol in the normal breast acts as a mitogen and together with progesterone and numerous other factors regulates growth, development, and differentiation of the mammary gland (Horwitz *et al.* 1985). It is beyond doubt that estrogens play also an important role in development and progression of human breast cancer. It may be recalled that if no estrogenic stimulation does occur, for example as a result of primary ovarian failure, the incidence of breast cancer is less than 1% of that in normal women (Lippman *et al.* 1986). There are strong indications that estrogens can function as carcinogens as well as regulators of tumor growth (Clark *et al.* 1989).

The mechanisms by which estrogens induce cell proliferation remain largely unresolved. However, circumstantial evidence supports the concept that epidermal growth factor (EGF) and its structural and functional homologue, transforming growth factor alpha (TGFα), play an important role as mediators in the estrogen effects (Lippman and Dickson 1989). Both EGF and TGFα are natural products of mammary cells but have also been detected in malignant mammary tumor tissue (Perroteau *et al.* 1986, Travers *et al.* 1988). There are indications that EGF functions principally via an autocrine mode, whereas TGFα acts locally via a paracrine or autocrine mechanism (reviewed by Velu 1990). It has been established many years ago that estrogens act via a highly specific intracellular receptor, the estrogen receptor (ER) (Jensen *et al.* 1982, Gorski *et al.* 1986) and, more recently, that EGF acts via a transmembrane receptor, the EGF receptor (EGFR) (reviewed by Carpenter 1987). It is of great interest that EGFR also binds TGFα and with a similar affinity as it binds EGF (Todaro *et al.* 1980, Massague *et al.* 1983).

From numerous studies it became clear that mammary tumor cells still contain functional ER and thereby may retain hormone responsiveness. In 60-80% of human breast tumor biopsies ER can be detected and the cells of these tumors theoretically could be responsive to endocrine treatments designed either to lower estrogen levels or to block its effects (anti-estrogen therapy) (McGuire *et al.* 1975). Unfortunately, only 50% of the ER positive tumors actually show a beneficial response. When the tumor is also positive for the progesterone receptor, PgR, this percentage is about 70% (Horwitz 1987). So, ER and PgR assays became important for predicting response to endocrine therapy as well as the clinical course of patients with early stage disease (Seibert and Lippman 1982). Gradually human breast tumor cells become hormone independent and the progression to this more autonomous state goes along with a loss of ER and PgR.

Studies in recent years have claimed and denied that quantification of EGFR could additionally provide prognostic information with respect to disease free survival and overall survival (see for reviews: Klijn *et al.* 1992, Koenders, doctoral thesis University of Nijmegen, 1992). A high EGFR content in human breast cancer biopsies has been reported to be associated with poor prognosis (Sainsbury *et al.* 1987). Furthermore, high EGFR levels have been found in association with low ER levels. However, the reported incidences of EGFR-positive cases varies between 15% and 91%, whereas the incidence of EGFR-negative ER-negative biopsies varies even between 8% and 35% (P.G. Koenders, doctoral thesis University of Nijmegen, 1992). These huge variations could be due to differences in methodology. Assay conditions differ widely with respect to: the preparation of the membrane fraction, the radioiodination procedure for preparing ^{125}I-EGF, the incubation conditions such as time and temperature, the ligand concentrations used in multiple point and single saturation

dose analysis, and finally the method of separation of receptor-bound and unbound ligand (Table 2.1). At least 20 studies involving EGFR assays reported so far differ from each other in one or more of these assay elements.
To restrict the variation in EGFR assay results between laboratories it was decided to establish an efficient analytical method which under rigid assay conditions gives reproducible results with high comparability between laboratories. In addition, since EGF receptor function depends in a dose-dependent manner on its ligand(s) (EGF, TGFα, and other related peptides), it was of interest to measure the overall level of EGFR ligand(s) in breast tumor cytosols. In the present thesis reproducible assays for EGFR and its ligand(s) were described (chapters 2 and 5 respectively).
The methods used for quantitation of EGFR employ radioiodinated EGF which is incubated with a membrane fraction of the mammary tumor. Radioiodination of a polypeptide can lead to oxidation of the ligand molecule and oxidation of ligands often has serious implications for the binding behavior towards the receptor (Bolton 1985). As a consequence, the affinity of radioligands for their receptor can become considerably less than the affinity of the unlabeled EGF. This important analytical issue, which is often disregarded (Kermode 1988), has been addressed thoroughly in chapter 4. It appeared that with three iodination methods, Chloramine T, Iodogen, and Iodo-beads, one obtains ^{125}I-EGF which, as was shown by HPLC analysis, is oxidized to a large extent and is not equivalent to unlabeled EGF in receptor binding. Unfortunately, these harsh oxidative iodination methods have been generally used in preparing radioligands for EGFR studies. It follows from the present study that there are other iodination procedures, iodine monochloride (ICl), Protag-125, and Enzymobeads, which can be employed for preparing nonoxidized ^{125}I-EGF. These mild iodination methods do not affect the binding characteristics of the ligand, while the specific radioactivities obtained are sufficiently high. Measurement of apparent dissociation constants and number of binding sites can be expected to be more reliable if there is equivalence in binding behavior between labeled and unlabeled ligand.
In quantitative binding studies using a single dose of radioligand, the concentration of the radioligand used is of paramount importance. The level of saturation of the receptor not only depends on the concentration of the ligand used, but also on the affinity of the receptor for the ligand. If in a tumor only one class of binding sites is present, e.g. with a Kd of 0.3 nM, a single point assay using a tracer concentration of 0.6 nM would saturate about 70% of the receptors. In another tumor with receptors with a Kd of 0.6 nM, the single point assay with the same tracer concentration would only result in a calculated receptor concentration of 50% of the receptors present. Increasing the amount of radioactive tracer is in this case only beneficial to a certain extent, because the relative difference between total binding and nonspecific binding decreases at increasing amounts of tracer. Since the Kd's observed in the analysis of different tumors differ widely, the use of a single point assay for routine EGFR analysis has little to commend it. The ligand concentrations used in different 'single point' studies varies between 0.1 nM and 10 nM. Results obtained in those studies therefore hardly can be compared with each other.
A wide range of ligand binding affinities have been reported, most of which have been derived by Scatchard analysis of binding data. Receptor populations with apparent Kd's varying between 0.1 nM and 1 nM are generally regarded as having a high affinity for the ligand. It may be emphasized that higher EGFR levels and apparent

Kd's are obtained at higher ligand concentration ranges if curvilinear Scatchard plots are observed and a linear regression analysis is employed. Curvilinear Scatchard plots are no unequivocal evidence for the presence of multiple classes of binding sites. Affinity difference between labeled and unlabeled ligand is one of the various other factors that can lead to curvilinear Scatchard plots (for reviews: Taylor 1975, Carpenter 1987, Kermode 1989). There is only one research group assessing EGFR in human breast cancer which demonstrates that the Scatchard plots obtained with tumors are curvilinear rather than straight lines (Nicholson *et al.* 1988). Nicholson *et al.* concluded from these observations (n=6) that two different types of binding sites are present, one with a Kd of about 0.6 nM, the high affinity binding site, and the second with a Kd of 11 nM, the low affinity binding site. It deserves attention that from an increasing number of biochemical EGFR studies performed on a great diversity of cell lines and tissues, evidence emerges that indeed high affinity (0.1-1 nM) and low affinity (3-10 nM) EGF binding sites exist. These studies include A431 cells (Lin *et al.* 1988), Hela cells (Fanger *et al.* 1984), human KB cells (King and Cuatrecasas 1982), as well as human thyroid glands (Kanamori *et al.* 1989), HCG producing tumors (Miyachi *et al.* 1990), human placentas (Hock and Hollenberg 1980, Chen *et al.* 1988), meningiomas (Weismann et al 1987), murine mammary epithelial cells (Taketani and Oka 1983), etcetera. Monoclonal antibodies directed to either high- or low-affinity receptors have been described, suggesting a conformational difference between the two receptor populations (Defize *et al.* 1988, Bellot *et al.* 1990). In our study, all tumors were analyzed by multiple point titration analysis. Albeit that a number of Scatchard plots were slightly curvilinear, the EGF binding data obtained with the breast tumor membranes could be fitted best according to a one binding site model. For routine EGFR analysis compromises had to be made and we used the most simple model for calculation of the binding data, yielding a relatively reliable estimate of the number of high affinity receptors (mean Kd about 0.5 nM) present in the membrane preparations. The apparent Kd ranged between 0.1 nM and 1.1 nM (95% of the EGFR positive tumors, n=341). It may be remarked that in the study of Nicholson *et al.* (Nicholson *et al.* 1988) the span of ligand concentrations used (0.15-10 nM) was definitely larger than the concentration range we used (0.1-2 nM). It might be that therefore in our study the low affinity binding site was not disclosed. Even in human placental membranes (HPM), which contain high levels of EGFR, no low affinity binding sites (Kd >3 nM) could be detected. In these particular experiments a more extended range of ^{125}I-EGF concentrations (0.02-7.7 nM) was used. Interestingly, however, it appeared that in HPM a small number of very high affinity binding sites were present (Kd 0.01 nM). Our observation is in agreement with a number of investigations in which it has been reported that certain carcinoma cells contain EGF binding sites with an extremely high binding affinity (Kd ≈0.01 nM) (Kawamoto *et al.* 1983, Winkler *et al.* 1989, Wada *et al.* 1990). It has even been suggested that the stimulatory effect of EGF might be mediated by these small amounts of very high affinity EGF receptors: occupancy of a small fraction of EGF receptors resulted in maximal stimulation of cell growth (Shechter *et al.* 1978). This important point has to be investigated in future experiments. Such investigations are the more imperative because there are strong indications that in a number of membrane fractions of breast tumors these extremely high affinity binding sites also are present. Measurement of those particular receptors requires a significant improvement of the sensitivity of the receptor assay. It is noteworthy to remark that HPLC purification of ^{125}I-EGF can yield tracers with very

high specific radioactivity and show equivalence in binding behavior between labeled and unlabeled ligand. The use of these ^{125}I-EGF preparations will certainly improve the sensitivity of the assay and may be beneficial in unravelling this phenomenon.
The ultimate EGFR assay developed included an improved procedure for the separation of receptor-bound from free ligand. A bound-free separation step introduces a disturbance of the equilibrium state, which is a drawback of all receptor binding studies. To achieve maximal recovery of receptor-bound ligand with a minimum of dissociation of the ligand-receptor complex, hydroxylapatite (HAP) was introduced in the receptor assay for the separation of receptor-bound from free ^{125}I-EGF. HAP is able to bind EGFR-containing membranes, leaving the nonbound ^{125}I-EGF in solution. The use of HAP permitted a rapid separation procedure. An evaluation of the procedure showed the use of HAP to be favorable over the use of centrifugation or filtration, separation procedures that have been generally used in studies on EGFR in breast cancer (chapter 3).
Recently, Falette *et al.* suggested that measurement of EGFR by the ligand binding assay only disclosed nonoccupied receptor sites (Falette *et al.* 1992). They claimed that local production of EGF-like factors results in a masking of binding sites. They propagated an acid treatment of the membrane preparation to dissociate the endogenously bound ligands before measuring the total, i.e. occupied and free, receptor sites. This interesting view was thoroughly tested in our laboratory and the results of these experiments are reported in chapter 3. hEGF and hTGFα were added to an HPM preparation and, after incubation, the membranes were collected by centrifugation. The number of binding sites measured in these membrane preparations was unaffected by this addition, neither was the apparent Kd. This result strongly indicates that the binding of these ligands to the receptor is completely reversible and this is in agreement with the competitive inhibition model. Furthermore it was shown that as a result of an acid treatment step, proteins in the tumor membrane preprations were eliminated without loss of receptor sites. So, expression of EGFR as a fraction of membrane protein content leads to higher values, which erroneously can be interpreted as increased receptor levels per se. The same effect as observed with acid treatment was achieved by a simple washing step at neutral pH.
The EGFR assay developed was easily applicable to the pellet fraction remaining following standard preparation of cytosols for routine assay of steroid hormone receptors. The developed HAP assay has been adopted by the European Organization for Research and Treatment of Cancer (EORTC) Receptor Study Group as standard for the measurement of EGFR in human derived tissue biopsy samples. From a pilot quality control trial it appeared that with the assay developed still a relatively large between-laboratory variation is observed, as compared to the small variation observed in the within assay experiments. This implies that extended quality control is highly desirable.
The radioreceptor assay (RRA) described in chapter 5 measures the overall level of EGFR ligand(s), i.e. all factors that compete with ^{125}I-EGF for binding to the EGF receptor. Apart from EGF, these factors include TGFα (Todaro *et al.* 1980), vaccinia growth factor (Stroobant *et al.* 1985), amphiregulin (Shoyab *et al.* 1989), and cripto (Ciardiello *et al.* 1991). In other words, no absolute specificity is obtained using this assay. Keeping this in mind, the overall levels of EGFR ligand(s) were assessed in human and mouse mammary tumor cytosols by RRA.
The levels of ER, PgR, EGFR, and EGFR ligand(s) were examined in a mouse

mammary tumor model. In this model progression of tumors to hormone independence is observed in serial transplants of tumors that are induced in ovariectomized female mice by continuous administration of estrone and progesterone (Sluyser and Van Nie 1974). The induced tumors are initially hormone dependent, but during transplantation they gradually become hormone independent. In the present collaborative study with Dr. Sluyser (Netherlands Cancer Institute) we observed, in concordance with their earlier studies (Sluyser *et al.* 1976), that hormone dependent (HD) tumors were positive for both ER and PgR, whereas hormone independent (HI) tumors contained only 30% of the ER concentration that was found in the HD tumors. PgR was undetectable in HI tumors.
Interestingly, HI tumors contained 2.5 to 3-fold higher EGFR levels than HD tumors. It may be recalled that high levels of EGFR in human breast cancer has been reported to be associated with poor prognosis (Sainsbury *et al.* 1987). Furthermore it was observed that the level of EGFR ligand(s) was three-fold higher in HD tumors than in HI tumors. With the specific radioimmunoassay (RIA) available for measuring mEGF, no EGF was detected in the tumor cytosols. This discrepancy between the RIA and the RRA results could be ascribed to the presence of the alternative ligand, TGFα, an interesting point which should be confirmed by a specific RIA for TGFα. The positive association observed between ER and EGFR ligand(s) is in view with an ER controlled production of the EGFR ligands (Dickson and Lippman 1987).
The low EGFR levels in HD tumors relative to HI tumors may have resulted from downregulation of the receptor by endogenous growth factors. It may be hypothesized that during progression to hormone independence this downregulation is less pronounced because of the lower level of ER. In this view, the increase in EGFR upon reaching hormone independence may therefore be a secondary effect rather than a key event in the progression to hormone independence in this mouse mammary tumor model, but future experiments are required to confirm this supposition.
It is considered as a very interesting and important point that the observations in the mouse mammary tumor model are in concert with the associations observed in human breast cancer - negative between ER and EGFR, negative between EGFR and EGFR ligand(s), positive between ER and EGFR ligand(s) -, as described in the doctoral thesis by P.G. Koenders ("Epidermal growth factor receptor and its ligand(s): associations with prognosis of patients with breast cancer", University of Nijmegen, 1992). The concordance between the results obtained in this in vivo mouse mammary tumor model and those obtained in human breast cancer makes this tumor model attractive for further clarifying the involvement of the "EGF"-EGFR pathway and its interactions with the steroid receptor pathway in the progression from hormone dependent to hormone independent breast cancer.

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Evaluation of ligand-binding parameters.

Interaction of a ligand with a binding protein at equilibrium may, according to the law of Mass Action, be represented as follows:

$$[F][N']/[B] = K \quad (1)$$

where:

[F] = free ligand concentration
[N'] = concentration of free, unoccupied binding sites
[B] = concentration of protein-bound ligand = concentration of occupied binding sites
K = equllibrium (dissociation) constant

Defining [N] as the total concentration of binding sites and rearranging yields:

$$[F][N-B] = K[B] \quad (2)$$

This expression can be rearranged either to:

$$[B]/[F] = -[B]/K + [N]/K \quad (3)$$

being the classical Scatchard [1] relation, or to:

$$[B] = [F][N]/(K+[F]) \quad (4)$$

which can be generalised to the m ligands, n orders of binding sites model according to Feldmann [2]. For one ligand and two orders of binding sites one obtains:

$$[B] = [F][N_1]/(K_1+[F]) + [F][N_2]/(K_2\{+[F]\}) \quad (5)$$

where now [B] represents the total concentration of bound ligand, summed over the two orders of binding sites.
So, in the case of a single ligand that binds to a single order of protein binding sites, transformation and plotting of binding data according to Scatchard ([B]/[F] vs [B], eq.3) yields, under the following conditions, a straight line:

1. Ligand and binding sites are in dynamic equilibrium
2. Quantitative separation of bound from free ligand, i.e. the separation technique must not affect the equilibrium, and free-in-bound or bound-in-free inclusion must not occur.

Under those ideal conditions, the slope of the line equals the affinity constant of the binding equilibrium, and the intercept on the abcissa reflects the total concentration of binding sites Thus, the above model is based on these two parameters equilibrium constant and binding sites concentration As will be shown below, it is convenient to express the equilibrium constant in terms of the dissociation constant as the latter has the dimensions of concentration

If the range of ligand concentrations employed in a binding experiment does not extend beyond a few percent of the dissociation constant, the bound/free ratio will be almost constant, resulting in a Scatchard curve that runs virtually parallel to the abcissa This is can be inferred from eq 4 by assuming that K is so high that [F] becomes negligible Now the equilibrium can be characterised by a single parameter, i e the dimensionless ratio of binding site concentration and dissociation constant [N]/K, which equals the constant bound/free ratio The binding sites are said to be "unsaturable", such in contrast with "saturable" sites that are characterised by two parameters [N] and K It is evident that "saturability" is a relative notion, depending on the range of ligand concentrations To be more precise, actually it is the range of free ligand concentrations that determines whether saturation is can be observed by the decrease of [B]/[F] with increasing ligand concentration or not

In receptor analysis it is usually assumed that the sample contains a combination of one order of saturable (two parameters) sites and one order of unsaturable (one parameter) sites (eq 5) Also it is assumed that the latter bears no relationship to the receptor itself ("non-specific" binding), but may arise from contaminating tissue or plasma, or experimental artefacts mimicking low-affinity binding Such a combination of two orders of binding activity theoretically leads to a non-linear Scatchard plot, from which the three parameters cannot readily be evaluated as can be done with either one-order-of-sites model Evidently, non-linear plots also will result with two orders of saturable binding sites (4-parameters model) or two saturable and one unsaturable order of sites (5-parameter model)

A graphical approach to analysis of binding data according to 3- and 4-parameter models was given by Rosenthal [3] This method requires that at least a few data points extend into the free ligand concentration range where the contribution of the higher affinity sites to total binding has become negligible A more widespread approach, which is actually a computed version of the Rosenthal method for the 3-parameter model, is by first estimating the third parameter by means of assessing ligand binding at excessive total ligand concentration, assuming that the contribution of saturable binding has become negligible Subsequently the binding data at lower ligand concentration are corrected for the unsaturable binding, thus yielding data that ideally should correspond to a two-parameter model that may be further analysed by determining slope and intercept With this method one runs the risk of underestimating the contribution of "unsaturable" binding within the regular concentration range, so that no complete linearisation is obtained Such underestimation may result from partial saturation occuring at extreme ligand concentrations in spite of the very low affinity To avoid this possibility, recently a new linearisation method for analysis of data according to a three-parameter model has been developed, in which no separate estimate of the third parameter is required (Van Zoelen [4])

Curve fitting

Fitting of linearised experimental binding data to a straight line, either "by hand" or by conventional unweighted linear least squares regression analysis gives unbiased estimates of dissociation constant and binding site concentration exclusively in the absence, or virtual absence of experimental error Both approaches presuppose an independent variable that is unaffected by experimental error and a dependent variable that is subject to a uniform,

normally distributed random error. These conditions, that also implicitly hold for "fitting by hand", are not fulfilled by any of the methods mentioned so far. In the Scatchard transformation, both the abcissa and ordinate variable are functions of the measured concentration of bound ligand that evidently is affected by random measurement error. The resulting error in the dependent variable (bound/free ratio) evidently is neither uniform nor independent. If the relation between the dependent variable and its variance is known, weighted linear regression analysis could be applied. However, this does not solve the problem of neither abcissa or ordinate being error-free. This latter problem only can be relieved to a certain extent by using one or more types of principal component analysis, of which orthogonal linear regression can be considered as a particular case, assuming that the measurement, or residual variances of both variables are equal and independent from each other. These assumptions never can never be fulfilled in a Scatchard plot because the variables are different functions of the same measurement result.
As it seems not to be feasible to obtain accurate, unbiased parameter estimates from linearised binding data, except in the virtual absence of measurement error, the logical alternative is to explore the possibilities of non-linear (weighted) regression analysis. The principal advantage of this approach is that it can be performed in such a way, that it closely reflects the reality of the experiment in which measured bound ligand (dependent variable with error) is a (non-linear) function of the (independent) most nearly error-free total ligand concentration. Moreover, binding models with more than two parameters are handled almost with the same ease as the simple two-parameter or one order of saturable binding sites model. The principle of non-linear regression analysis is the same as for linear regression, in that it aimes at parameter estimates that yield a minimum value for the sum of squared deviations of the measured data points from the curve that is based on these parameter estimates. In weighted regression analysis, the deviations are expressed in terms of the expected standard deviation of the measurement signal at each point. The sum of squares of these deviations from the fitted curve is approximately chi-square-distributed. With linear regression analysis, the chi-square minimum with its corresponding parameter values can be calculated right away, whereas non-linear models require an iterative procedure according to a suitable algorithm (e.g. Marquardt [5]) yielding succesive parameter estimates such that the minimum chi-square value is rapidly approached. If the expected variances of the measurement points cannot be evaluated experimentally, e.g. if no replicates are available, an assumption concerning the relation between the magnitude of the measurement signal and its error has to be made. In ligand-binding analysis using radioactively labelled ligand with low count rates, a reasonable assumption is a direct proportionality between variance and signal height.

Choice of the binding model.

In regression analysis a rule of thumb is, that the number of parameters to be estimated from a particular set of data should not exceed half the number of available data points, so that the number of remaining degrees of freedom will not be less than the number of estimated parameters. Thus, when eight points are available, one has the choice between two, three and four parameters to be evaluated. The chi-square values obtained from the three different curve-fittings are suited as a measure of goodness of fit. The model that is most appropriate for the data gives the lowest chi-square value. Chi-square values can be compared by means of the F-tables. E.g. with eight data points the chi-square for a four parameter fit must be 4.53 times as high as for a two-parameter fit in order to reach significance at the $p<0.05$ level. In the case there is no significant difference between models one should adhere to the simpler one for description of the data.

Regression analysis also can be applied to data that have been corrected for "non-specific binding". Thus, it is possible to impose a model that has one parameter in addition to the number that is allowed from the number of measurement points. E.g. nine data points normally only allows fitting of four parameters. By using the highest dose point for estimating a fith parameter (constant bound/free ratio of unsaturable or non-specific binding) and subsequently correcting the other points one can evaluate the data according to a five parameter model in which one parameter is estimated by direct measurement and the other four by curve-fitting.

Selection of the measurement range.

When the supply of test material is limited, the number of data points has to be limited accordingly, and selecting the proper range of ligand concentrations becomes critical.
It is extremely difficult to predict by calculation which dose points will yield the most significant information. When the free ligand concentration ranges from 0.1 to 10 times the dissociation constant of the receptor binding sites, saturation (i.e. [B]/[N]) of these sites runs from 10 to 90 % , as can be calulated from eq. 4. Without taking into account the influence of the measurement error it is intuitively felt that even distribution (e.g. free ligand concentrations of 0.11, 0.25, 0.43, 0.67, 1, 1.5, 2.33, 4 and 9 times the dissociation constant leads to saturation from 10 to 90% with regular 10% intervals) within this range gives the most significant information.
When the expected dissociation constant may vary by one order of magnitude one could aim at a range that gives 10% saturation of the low estimate to 90% saturation for the high estimate, implying from 1% for the high to 99% for the low estimate.

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Regulatie van groei van cellen en weefsels is een uitermate gecompliceerd proces waarin vele hormonen en zogenaamde groeifactoren een sturende rol spelen. Wil een hormoon of een groeifactor een effect hebben op een cellulair proces, dan dient de onderhavige cel receptoren te bezitten voor dat specifieke hormoon of die specifieke groeifactor.
Kwalitatieve en kwantitatieve metingen van receptoren is van belang gebleken om inzicht te verkrijgen in de wijze waarop de groei van normale cellen en weefsels door hormonen en groeifactoren wordt gereguleerd. Zulke inzichten zullen ongetwijfeld een positieve betekenis hebben voor het opsporen van defecten in de groeiregulatie. Een defect in de groeiregulatie kan als gevolg hebben dat er kwaadaardige tumoren ontstaan waarvan de groei niet beteugeld is.
Het is in de laatste jaren steeds duidelijker geworden dat de geslachtshormonen oestradiol (E) en progesteron (Pg), in samenspel met onder andere de epidermale groeifactor (EGF), de groei, ontwikkeling en differentiatie van de borstklier reguleren. Het lijdt geen twijfel dat oestradiol ook een belangrijke rol speelt in de ontwikkeling en progressie van borstkanker. De aanwezigheid van receptoren voor de geslachtshormonen E (ER) en Pg (PgR) in een borsttumor is een aanwijzing voor hormoon-gevoeligheid van de tumor en wordt als leidraad gebruikt bij het maken van de keuze voor de juiste therapie voor patiënten met borstkanker. Helaas kan op deze wijze niet bij alle patiënten de reaktie op hormonale therapie betrouwbaar worden voorspeld. Naast de analyse van de steroidhormoon-receptoren is analyse van aar vullende prognostische factoren essentieel.
De wijze waarop oestrogenen celproliferatie kunnen induceren is slechts zeer ten dele bekend. Er zijn in de laatste jaren echter steeds meer aanwijzingen verkregen, dat EGF een mediërende functie heeft bij oestradiol effecten. Als gevolg van het signaal dat oestradiol afgeeft aan de cel, wordt door bepaalde cellen van de borstklier EGF of EGF-achtige factoren (EGFR liganden) geproduceerd. Deze liganden blijken in staat om de cellen die deze liganden produceren te stimuleren (autocriene regulering). Bovendien kunnen deze liganden als stimulatoren werken op naburige cellen (paracriene regulering). Receptoren voor oestradiol zijn in het celplasma gelocaliseerd, receptoren voor EGF bevinden zich in de celmembraan. De ontwikkeling en evaluatie van kwantitatieve analyses van deze celmembraan geassocieerde EGFR en van EGFR liganden zijn onderwerpen van studie geweest waarvan de resultaten in deze dissertatie zijn beschreven.
Ondanks verschillende onderzoekingen is de betrokkenheid van EGFR bij het ontstaan en de progressie van borstkanker niet eenduidig gebleken. Het percentage EGFR-positieve borsttumoren dat wordt gerapporteerd in de literatuur varieert zeer sterk (van 15% tot 91%), evenals de hoeveelheden aanwezig EGFR (hoofdstuk 1).

Deze grote variatie aan EGFR waarden resulteert in zeer controversiële resultaten m.b.t. de prognostische waarde van EGFR bij borstkanker. Het ontbreken van een algemeen geaccepteerde, betrouwbare methode voor het meten van EGFR zou hier zeker debet aan kunnen zijn. De Europese Organisatie voor Onderzoek en Behandeling van Kanker (EORTC) heeft ons laboratorium aangespoord een methode te ontwikkelen voor het betrouwbaar meten van EGFR. De ontwikkeling, optimalisering, en evaluatie van deze nieuw geïntroduceerde methode zijn uitvoerig beschreven in de hoofdstukken 2 en 3. De huidige methode is inmiddels door de EORTC geaccepteerd om als standaardmethode te fungeren voor de meting van EGFR in menselijke weefsels.

Het principe van de ontwikkelde receptormeting berust op de binding van radioactief gemerkt EGF (^{125}I-EGF), aan geïsoleerde membranen van een tumor, waarna receptor-gebonden ^{125}I-EGF en niet gebonden ^{125}I-EGF worden gescheiden middels hydroxylapatite (HAP). Het radioaktief merken (joderen) van EGF kan tot gevolg hebben dat de interactie van de ligand met de receptor ongunstig wordt beïnvloedt. Echter, om een zo betrouwbaar mogelijke bepaling te garanderen dient het ^{125}I-EGF dezelfde bindingseigenschappen te bezitten als het niet gejodeerde EGF. Dit belangrijke analytische aspect is intensief onderzocht in hoofdstuk 4. Uit de hier beschreven studie bleek dat gangbare joderingsmethodes (Chloramine T, Iodogen, Iodo-beads) leiden tot oxidatie van ^{125}I-EGF. De interactie van deze geoxideerde producten met EGFR is niet identiek aan de interactie van het normale EGF met de receptor. Het zijn juist deze geoxideerde liganden die over het algemeen gebruikt worden in studies naar de rol van EGFR bij borstkanker. Tevens is gebleken dat m.b.v. mildere joderingsmethodes (ICI, Protag-125, Enzymobeads) ^{125}I-EGF preparaten kunnen worden verkregen die wel identieke bindingseigenschappen vertonen als die van het ongemerkte EGF. Het gebruik van deze niet geoxideerde ^{125}I-EGF preparaten in EGFR bindingsstudies leidt tot meer betrouwbare EGFR metingen, hetgeen voor de bestudering van de rol van EGFR bij borstkanker van belang is.

Niet alleen EGF is in staat om aan EGFR te binden. Ook verwante polypeptiden, waaronder de transformerende groeifactor alfa (TGFα) zijn in staat om aan EGFR te binden met als gevolg een groeistimulerend effect. In tegenstelling tot het overvloedige aantal studies naar de rol van EGFR in borstkanker, is er sporadisch onderzoek verricht naar de rol van EGFR ligand(en) bij deze aandoening. Gebruikmakend van een EGFR-bevattend humaan placenta preparaat en het principe van de HAP-assay kon een radioreceptor assay (RRA) worden ontwikkeld waarmee de combinatie van deze EGFR ligand(en) in het weefsel van borsttumoren kon worden bepaald. Verder werd m.b.v. een tegen muize-EGF opgewekt antilichaam een radioimmunoassay (RIA) ontwikkeld waarmee specifiek EGF kon worden bepaald in tumoren afkomstig van muizen. De RRA en de RIA zijn beschreven in hoofdstuk 5.

De in dit proefschrift beschreven methoden werden toegepast zowel in een klinische als in een experimentele studie. De resultaten van de klinische studie bij patiënten met een mammacarcinoom zijn beschreven in het proefschrift van P.G. Koenders ("Epidermal growth factor receptor and its ligand(s): associations with prognosis in patients with breast cancer, Universiteit Nijmegen, 1992), dat tegelijkertijd met het onderhavige proefschrift verschijnt. De resultaten van de experimentele studie, waarbij muize-mammatumoren werden geanalyseerd, zijn beschreven in hoofdstuk 6 van dit proefschrift. Het betreft hier hormoon-afhankelijke (HA) mammatumoren die door seriële transplantaties hormoon-onafhankelijk (HO) zijn geworden. De HA-tumoren

bleken positief voor ER en PgR, terwijl de HO-tumoren slechts 30% van de oorspronkelijke ER concentratie bevatten en PgR-negatief waren geworden, hetgeen de progressie naar HO status van de tumoren illustreert. De concentratie EGFR-ligand(en) in de HA-tumoren, gemeten met de RRA, was drie maal zo hoog als in de HO-tumoren, wat erop zou kunnen wijzen dat ook in dit proefdiermodel de synthese van EGF en/of TGFα gereguleerd wordt door ER. Met de specifieke RIA kon echter in geen van de tumoren EGF worden aangetoond. Dit wijst erop dat TGFα de ligand is die aanwezig in deze tumoren. Een specifieke bepaling voor TGFα zal dit in toekomstige experimenten moeten uitwijzen.
Interessant was de waarneming dat de onderzochte HO-tumoren 2,5 tot 3 maal zoveel EGFR bevatten dan de HA-tumoren. In dit verband dient vermeld te worden dat bij menselijke borsttumoren hoge EGFR waarden geassocieerd zijn met een slechte prognose van de patiënt. Een zeer belangrijk punt in deze is het feit dat de associaties tussen de verschillende receptoren en factoren die werden gevonden in dit muize-tumormodel overeenkomen met de associaties die gevonden werden in de klinische studie bij patiënten met borstkanker, zoals beschreven in het bovengenoemde proefschrift van P.G. Koenders. Deze analogie en het feit dat kennelijk zowel bij de mens als bij de muis uiteindelijk hormoon-onafhankelijkheid optreedt, maken dit muize-tumormodel zeer de moeite waard om de progressie van hormoon-afhankelijkheid naar hormoon-onafhankelijkheid, en de eventuele betrokkenheid van EGFR en zijn liganden daarbij, nader te bestuderen. Dergelijk onderzoek is van belang om in de toekomst borstkanker beter te kunnen behandelen.

De schrijver van dit proefschrift werd op 8 augustus 1965 te Harbrinkhoek in de gemeente Tubbergen geboren. Na het behalen van zijn HAVO-diploma aan het Pius-X-College te Almelo in 1982, volgde hij het Hoger Laboratorium Onderwijs, afstudeerrichting klinische chemie, aan de HTS/SLP te Hengelo. Het diploma werd in 1986 behaald en datzelfde jaar begon hij met de studie scheikunde met hoofdvak biochemie aan de Rijksuniversiteit te Groningen. In augustus 1989 werd het doctoraal examen "met genoegen" behaald. Vanaf 1 september 1989 is hij als assistent in opleiding tot onderzoeker werkzaam op de afdeling Experimentele en Chemische Endocrinologie (hoofd: prof.dr. Th.J. Benraad) van het Academisch Ziekenhuis St. Radboud te Nijmegen. Aldaar werd het onderzoek verricht dat tot dit proefschrift heeft geleid.

Het is vanzelfsprekend niet uitsluitend mijn eigen inbreng geweest die geleid heeft tot dit proefschrift. Gaarne wil ik dan ook mijn erkentelijkheid uitspreken aan diegenen die mij in de gelegenheid hebben gesteld dit onderzoek te doen en diegenen die hebben bijgedragen aan de totstandkoming van dit proefschrift.
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